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DISSERTATION APPROVAL
FOR THE DOCTORAL DISSERTATION
IN THE MOLECULAR AND CELL BIOLOGY
GRADUATE PROGRAM

Title of Dissertation: "Critical Role of CD8 T Cells in Mediating Sex-Based Differences in a Murine Model of Lupus"

Name of Candidate: Anthony D. Foster
Doctor of Philosophy Degree
September 4, 2009

DISSERTATION AND ABSTRACT APPROVED:

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Report Documentation Page

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
21 AUG 2009		**00-00-2009 to 00-00-2009**

4. TITLE AND SUBTITLE	5a. CONTRACT NUMBER
Critical Role Of CD8 T Cells In Mediating Sex-Based Differences In A Murine Model Of Lupus	5b. GRANT NUMBER
	5c. PROGRAM ELEMENT NUMBER
6. AUTHOR(S)	5d. PROJECT NUMBER
	5e. TASK NUMBER
	5f. WORK UNIT NUMBER
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) **Uniformed Services University Of The Health Science,4301 Jones Bridge Rd,Bethesda,MD,20814**	8. PERFORMING ORGANIZATION REPORT NUMBER
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)	10. SPONSOR/MONITOR'S ACRONYM(S)
	11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Systemic lupus erythematosus (SLE) is a chronic autoimmune disease with a wellcharacterized but poorly understood female predominance in its prevalence. The DBA into B6D2F1 (DBAF1) model of chronic graft versus host disease (cGVHD) exhibits a lupus‐like glomerulnephritis (GN) that results from immune complex deposition in the kidneys. Following the transfer of unfractioned DBA splenocytes into an unirradiated BDF1 host, parental strain DBA CD4 T cells recognize the B6 parental strain MHC II on all host B cells, which subsequently become activated and produce large amounts of anti‐DNA and anti‐nuclear autoantibodies. The subsequent renal disease has a strong female predilection in disease severity, comparable to SLE, making it a useful model for the investigation of sex‐based differences in lupus.

15. SUBJECT TERMS

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			
unclassified	**unclassified**	**unclassified**	**Same as Report (SAR)**	**144**	

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MOLECULAR AND CELL BIOLOGY PROGRAM
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September 4, 2009

Abstract

Title of dissertation: “Critical Role of CD8 T Cells in Mediating Sex-Based Differences in a Murine Model of Lupus”

Anthony D. Foster, Doctor of Philosophy, 2009

Thesis Directed by: Charles S. Via, M.D.

Department of Pathology

Systemic lupus erythematosus (SLE) is a chronic autoimmune disease with a well-characterized but poorly understood female predominance in its prevalence. The DBA into B6D2F1 (DBA→F1) model of chronic graft versus host disease (cGVHD) exhibits a lupus-like glomerulnephritis (GN) that results from immune complex deposition in the kidneys. Following the transfer of unfractioned DBA splenocytes into an unirradiated BDF1 host, parental strain DBA CD4 T cells recognize the B6 parental strain MHC II on all host B cells, which subsequently become activated and produce large amounts of anti-DNA and anti-nuclear autoantibodies. The subsequent renal disease has a strong female predilection in disease severity, comparable to SLE, making it a useful model for the investigation of sex-based differences in lupus.

In contrast to the necessary and sufficient role of donor CD4 T cells in inducing lupus-like disease, donor CD8 T cells are not thought to play a role in the DBA→F1 model. To confirm the

role of donor CD8 T cells we injected age- and sex-matched F1 hosts with either unfractionated DBA splenocytes (CD8 intact→F1) or CD8-depleted donor inocula (CD8 depleted→F1). As expected, lupus-like GN was observed in all injection groups at 13 weeks post injection and sex based differences seen in CD8 intact→F1 groups. Surprisingly, sex-based differences were lost in CD8-depleted→F1 groups based on clinical and serological evidence of nephrotic syndrome. Electron microscopy confirmed a severe membranous GN in CD8-intact female into female transfers (f→F) mice that was reduced in CD8 depleted f→F mice. Flow cytometry analysis showed increased numbers of splenic host $CD4^{+}ICOS^{Hi}$ T follicular helper cells (T_{FH}) in CD8 intact f→F vs. m→M, which was lost in CD8-depleted→F1 groups and a corresponding reduction in IL-21 gene expression. CD8 depletion prior to injection prevented sex based differences by reducing CD4 T cell help to B cells in f→F mice that displayed reduced disease severity, and through a slight worsening of disease in m→M mice.

Short term assays were performed to determine the role of donor CD8 T cells in the DBA→F1 model where severity of disease parallels the strength of the donor CD4 response. The day 14 engraftment of donor CD4 T cells in the DBA→F1 model is 2-3 fold greater in f→F mice vs. m→M mice and acts as a surrogate marker for long-term disease severity. Similar to our week 13 findings, CD8 depletion prior to injection prevented the 2-3fold increase in donor CD4 engraftment at day 14. CD8 depleted resulted in a significant reduction in f→F donor CD4 engraftment and a slight (but insignificant) increase in m→M engraftment in CD8 depleted→F1 mice. Kinetic analysis of days 8-12 revealed a stronger host anti-graft (HVG) response in m→M mice, which was mostly dependent on the presence of donor CD8. The longer engraftment of donor CD8 in females was associated with sustained donor CD4 engraftment and donor CD4 proliferation was found to be enhanced in both sexes by the presence of donor CD8 T cells based on the expression of KI67. We conclude that donor CD8 T cells shape sex based

differences in the DBA→F1 model by inducing a stronger HVG response in m→M mice and by enhancing donor CD4 proliferation in f→F by persisting longer in female hosts.

Title Page

"Critical Role of CD8 T Cells in Mediating Sex-Based Differences in a Murine Model of Lupus"

By

Anthony David Foster

Dissertation submitted to the Faculty in the Molecular and Cellular Biology Program of the Uniformed Services University of the Health Sciences in partial fulfillment of the requirements of Doctor of Philosophy 2009

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Chapter 1: Introduction

Systemic lupus erythematosus (SLE) is a chronic inflammatory autoimmune disease resulting from multi-system immune complex mediated organ damage. A well-recognized but poorly understood feature of this disease is a strong female predominance in its prevalence. This includes a female to male ratio of approximately 9:1 during child-bearing years (1). This ratio is reduced in pre-adolescent and post-menopausal females, suggesting a critical role for sex hormones. The role of estrogen and other hormones has been studied extensively (2-5) in both lupus patients and in animal models. In summary, these studies have shown that female sex hormones including estrogen increase the incidence and severity of disease whereas loss of estrogen and/or the addition of androgens alleviate or reduce clinical features of the disease. To date it remains unclear what cellular events distinguish the female immune response from the male immune response resulting in this sex-based disparity.

Pathogenesis

Organ damage in both human SLE and animal models is thought to result from the production of anti-nuclear autoantibodies and subsequent immune complex (IC) formation and deposition. IC deposition in SLE patients typically involves multiple organ systems, which results in heterogeneity of clinical manifestations. Renal failure is a common feature of the human disease and is also seen in some murine lupus models (2, 3). Lupus nephritis results from IC deposition in the kidneys resulting in glomerular damage and inflammation. IC formation occurs either locally (*in situ*) or in circulation,

followed by glomerular deposition (4). The cellular and molecular events that result in glomerular damage involve interaction between multiple cellular populations, including B cells, T cells, and macrophages, as well as complement, autoantibodies, and inflammatory cytokines.

Mechanisms of Tissue Damage (molecular pathogenesis)

The precise mechanism of pathogenesis in lupus remains controversial, and may vary between individuals. Pathogenic autoantibodies may be able to induce tissue damage independently, as has been seen following transfer into healthy mice (5, 6). However, there is a consensus that immune complex (IC) formation and deposition is a major mechanism in lupus pathogenesis (4). Importantly, not all IC are pathogenic. The pathogenic capability of a given IC is determined by several factors, including size, quantity, tissue tropism, charge, and the ability of the innate immune system to clear IC and apoptotic bodies (reviewed in (7)). The clearance of apoptotic bodies and IC is defective in SLE, resulting in accumulation of available nuclear material and increased exposure to autoreactive lymphocytes (8). In normal individuals, large IC deposit transiently in the mesangium and are cleared by phagocytic monocytes and macrophages whereas small IC are excreted in urine (7). Intermediate sized, soluble IC are more likely to cause damage. However, they must be in sufficient quantity to overwhelm the clearing mechanisms of phagocytic cells.

Autoantibodies may cause renal tissue damage directly. Anti-DNA antibodies can bind to nuclear material trapped in the glomerular basement membrane, and may also bind directly to non DNA antigens such as laminin, heparin-sulfate, collagen type IV, ribosomal P protein, or α-actinin (reviewed in (9)). Those that bind to the surface of cells

may induce complement-mediated cytotoxicity (9). Additionally, some anti-DNA antibodies have been shown capable of internalization within cells and subsequent induction of apoptosis (10).

Complement

There is a paradoxical role for complement in lupus pathogenesis. On one hand, active disease is associated with activation of the classical complement pathway (11, 12). It is generally believed that autoantibodies within circulating immune complexes fix and activate complement, contributing to tissue damage. Similarly, in murine models of the disease IgG2a autoantibodies are of particular importance to disease pathology because of their ability to fix complement (13). Conversely, it has been shown that individuals with a homozygous deficiency in components of the classical pathway of complement activation are at increased risk of developing SLE. Specifically, individuals who are homozygous deficient for the C1 complex proteins (C1q, C1r, or C1s), and C4 have a greater prevalence of disease and develop a more severe disease (14, 15). Individuals deficient for C2 are at greater risk for SLE, though less so than those deficient in C1 or C4 (16). Additionally, other health conditions that result in very low complement levels may predispose individuals to SLE (12). It is not clear how complement can have these two contradictory roles in lupus pathogenesis. One possible explanation is that complement activation facilitates disease activity as an effector of inflammation. However, deficiency in components of the classical pathway probably increases the likelihood of disease by impeding the normal clearance of apoptotic bodies, which provide much of the antigen in SLE. As such, the increased exposure of autoreactive T

cells and B cells to their respective antigen increases the likelihood of an autoimmune response.

Autoantibodies

The hallmark of SLE pathogenesis is the production of pathogenic autoantibodies recognizing nucleic acid and other nuclear targets (17). While healthy individuals normally produce low levels of antibodies that recognize self-targets, pathogenic autoantibodies differ from these in several critical ways. First, non-pathogenic autoantibodies consist mostly of the IgM isotype and are generated independently of T cell help. They also cross-react with a variety of targets to which they have a low avidity (18). By contrast, pathogenic antibodies in lupus consist largely of the IgG isotype, indicating the availability of T cell help to B cells for class switching (19). They also bind with high avidity to specific targets (18). The ability to fix complement appears to be important as well (12, 19). Finally, pathogenic autoantibodies in lupus are produced in large quantities as a result of polyclonal B cell activation and hyperactivity (19).

It is helpful here to briefly review the process of antibody assembly. There are three critical phases of antibody assembly that determine the specificity and function of the resulting immunoglobulin (Ig) (reviewed in (20)). The first involves the stochastic recombination of gene exons that code for the variable (binding) region of the antibody. The variable (V), diversity (D), and joining (J) gene segments are assembled under the direction of the recombination activating genes (RAG) 1 and 2 during B cell development in the process of VDJ recombination. Additional genetic variability in the binding region of the antibody is achieved through somatic hypermutation. During this process, a high rate of mutation is introduced into the sequences encoding the heavy and light chain

variable regions that have previously undergone successful VDJ recombination. Finally, the heavy chain constant region determines the function of the resulting Ig, which may be one of several isotypes. Class switching and somatic hypermutation are controlled by activation induced cytidine deaminase, which is only expressed in germinal center B cells that have been activated (21). Importantly, during lymphocyte development, those B cells that bind too strongly to self-targets are selected for clonal deletion in the process of central tolerance (22, 23). Despite this process however, B cell clones that produce Ig which bind to self-target with low affinity do survive and produce the normal repertoire of autoreactive Ig described above. For these cells, potentially pathogenic responses to self are prevented by multiple mechanisms of peripheral tolerance.

Studies on lupus antibodies have demonstrated that they are highly mutated, especially in the hypervariable regions of both the heavy and light chains (18, 24). This indicates that such antibodies arise from the normal process of somatic hypermutation and affinity maturation and not from defects in central tolerance. As described above, somatic hypermutation occurs after the process of central tolerance, and is dependent on B cell activation and $CD4^+$ T cell help. Therefore the break in tolerance that occurs in SLE is in peripheral tolerance. It is not known how peripheral tolerance is broken is SLE. However, once autoreactive B cells receive T cell help and bind their respective antigens, subsequent generations of autoreactive B cells then develop greater specificity for nuclear targets through the normal process of affinity maturation (24, 25). Additional autoantigens are targeted through the process of epitope spreading where T cells and B cells specific for multiple epitopes on a complex of autoantigens coactivate, resulting in a gradual change in the specificity of autoantibodies (10).

While there is a range of autoantibody specificities seen in lupus, there is a common trend towards anti-nuclear targets including ds-DNA (26-28). It is generally believed that B cell hyperactivity in lupus is not random or targeted to any ubiquitous self-antigen. Studies in both lupus patients and murine models have shown remarkable similarity in the pool of antigens targeted (26-29). The common underlying feature of autoantigens targeted in SLE is their presence in the blebs of apoptotic cells (30). The normal process of T cell help to B cells appears to be intact, but dysregulated. Therefore the etiology of pathogenic antibodies in SLE is believed to result from abnormalities in B cell and T cell function.

B Cells

B cell hyperactivity is a critical feature of lupus pathogenesis and abnormalities have been found in both lupus patients and murine models of the disease. The excessive production of anti-DNA and anti-nuclear autoantibodies results from inherent B cell defects in lupus patients (31). Normally, tolerance of B cells to self-antigen is maintained by several mechanisms including deletion of autoreactive B cell clones, anergy, B cell receptor editing, and apoptosis among others (15, 16). To become activated, those mature self-reactive B cells that survive central tolerance to enter the periphery still require help, or signal 2, from antigen specific $CD4^+$ T cells (32). In both human lupus and murine models of the disease one or more of these steps is defective. As a result, lupus patients exhibit increased numbers of mature B cells as well as aberrant survival of autoreactive B cells that are resistant to apoptosis and other inhibitory mechanisms, and secrete large amounts of high affinity IgG antibodies to self antigen (31). However, these findings are based on patients or animal models with ongoing disease. The B cell abnormalities

observed might not reflect pre-existing abnormalities that actively participated in the initiation of disease. Rather, they may be the consequence of an already dysregulated immune system following the onset of disease.

There are three main subpopulations of circulating B cells: B1 B cells, marginal zone (MZ) B cells, and follicular B cells. B1 B cells secrete mostly IgM antibodies. MZ B cells are responsible for most T cell independent B cell responses (33). It has been suggested that autoreactive B cells that are not corrected by BCR editing are sequestered in the marginal zone (33). Naturally occurring anti-DNA B cells that act as precursors to autoantibody secreting plasma cells were found in the MZ in murine SLE (34). However, as mentioned previously, pathogenic autoantibodies in SLE are highly mutated in the variable binding region indicating a requirement for T cell help. While both B1 and MZ B cells are able to produce anti-DNA antibodies it is likely that the large amounts of anti-DNA IgG antibodies that characterize lupus are made by follicular B cells. This subpopulation of B cells can be found in close proximity to T cells in lymphoid tissue and are largely responsible for T cell dependent B cell responses (35).

The interaction between CD40 on B cells and its ligand CD154, expressed on both T cells and B cells, is important in lupus. These surface proteins play a critical role in germinal center reactions and the formation of plasma cells. Plasma cells secrete large amounts of antibody and are elevated in numbers in lupus patients (36). Long-lived plasma cells are formed during T cell dependent responses within germinal centers, whereas short-lived plasmablasts are formed during T independent responses (37). Both arise from B cells and are generated in secondary lymphoid organs. A critical step in the differentiation of plasma cells is interaction between CD154 and CD40 within germinal

centers (37). T cell signaling is a necessary step in the initial phase of B cell differentiation, and is antigen specific (38). In addition to Class II interactions with the T cell receptor, CD40 expressed on the surface of B cells interacts with its ligand CD154 expressed on T cells. This stimulates the expression of CD154 on B cells and allows for subsequent homotypic interactions between CD40 and CD154 on B cells (39). Peripheral B cells (and T cells) from patients with active SLE constitutively express CD154 (37). Conversely, in normal individuals, peripheral blood T and B cells generally do not express CD154 (39, 40).

B cells from both lupus patients and murine models of lupus exhibit aberrant survival and resistance to apoptosis (41, 42). This is consistent with increased numbers of circulating mature B cells commonly observed (7). Several mechanisms have been proposed using murine models to explain B cell survival in lupus, including alterations in signaling for Fas/FasL interaction, Bcl-2, and Bim (43-46). Impaired B cell death is of particular relevance to this project as it is a proposed mechanism for the female predominance of disease. Using a mouse model transgenic for an anti-dsDNA B cell receptor (BCR) it has been demonstrated that estrogen treatment rescues autoreactive B cells from deletion by normal mechanisms of tolerance induction (47). B cell survival was associated with upregulation of the anti-apoptotic marker Bcl-2 in this study. This group therefore proposes that sex based differences in lupus are due, at least in part, to ineffective downregulation of autoreactive B cells via apoptosis as a result of exposure to estrogen. Estrogen has been clearly identified as a potent regulator of immune function, as will be discussed later, and sex based differences in autoimmunity are believed to be related to sex hormones. However, estrogen treated B cells that were rescued from cell

death were shown to develop preferentially into MZ B cells (48). Marginal zone localization would limit interaction with T cells, which a critical feature in lupus pathogenesis. Limited T cell interaction would also prevent the process of epitope spreading common in lupus (49).

T Cell Abnormalities

The T cell dependent humoral antibody response begins with activation of naïve $CD4^+$ T cells following interaction between the T cell receptor (TCR) and Class II on an antigen presenting cell (APC). This interaction is specific to the antigen peptide presented by Class II and additionally requires costimulation through CD80 or CD86 expressed on APC and CD28 expressed on the T cell. Once activated, the $CD4^+$ T cell can proliferate and provide T cell help to B cells that similarly present cognate peptide in the context of Class II (38). To prevent the generation of autoreactive T cells, thymic development includes a process of central tolerance similar to that seen in B cells (50). As with B cells, $CD4^+$ (and $CD8^+$) T cells with moderate to low affinity for self-antigens do survive to enter the periphery. Mechanisms of peripheral tolerance, including $CD4^+Foxp3^+$ regulatory T cells, prevent potentially pathogenic T cell responses to self-targets from occurring. However in SLE, peripheral tolerance is broken through mechanisms that are as yet unknown. The resulting immune dysregulation includes multiple T cell abnormalities. As with B cell abnormalities in lupus, the T cell abnormalities that have been reported in lupus may be causal of the disease or may also be secondary effects of a deranged immune system.

T Cell Driven Disease:

Pathogenic B cell responses in lupus are dependent on help from $CD4^+$ T cells. As such, $CD4^+$ T cells are thought to play a critical role in SLE pathogenesis and have been shown to be necessary and sufficient for lupus like disease in murine models (17, 18). As mentioned previously, pathogenic antibodies in lupus typically have an IgG isotype and a variable binding region that has undergone extensive somatic hypermutation. Both ofthese features are T cell dependent (19). The process of epitope spreading is also believed to be T cell dependent (51). Consistent with these disease features, T helper function is exaggerated in lupus and interruption of T cell help to B cells improves disease (52). Surprisingly, the number of peripheral blood $CD4^+$ T cells in SLE patients is reduced (7). This is due, at least in part, to the production of anti-lymphocyte antibodies and correlates with disease activity (53). However the number of peripheral blood T cells may be less relevant than the number of T cells present in lymphoid organs where they interact with B cells.

Regulation and Tolerance

Several mechanisms of peripheral tolerance prevent T cells with low affinity for autoantigen from responding and potentially causing damage. These include active suppression by $CD4^+Foxp3^+$ regulatory T cells (Treg), activation induced cell death, anergy, and the production of suppressive cytokines including IL-10 and TGF (reviewed in (52)). Peripheral blood Tregs are reduced during active SLE compared with normal controls (54). Whether there is a similar reduction in the number of Tregs found in secondary lymphoid tissue where autoreactive T and B cells interact is not known. A class of $CD8^+$ T cells ($CD8^+$ Ts) capable of suppressing $CD4^+$ T cell activity by multiple

mechanisms has been described previously in murine models (55). $CD8^+$ Ts cells in the NZB/NZW F1 model are capable of suppressing lupus like disease but become defective with age (56). Similarly, circulating $CD8^+$ T cells from lupus patients have impaired ability to suppress $CD4^+$ T cell proliferation compared to normal controls (57, 58). Dendritic cells are capable of suppressing T cell responses and are thought to be either tolerogenic or activating based on their cytokine expression (59). This mechanism of peripheral tolerance is also defective in SLE. In summary, the known mechanisms of peripheral tolerance are incapable of maintaining tolerance in SLE. However it is not known how peripheral tolerance is initially broken, resulting in this condition.

Apoptosis

Regulation of T cell apoptosis is abnormal in both SLE and murine models. T cells from some SLE patients are defective in activation induced cell death, possibly due to decreased TNF-α synthesis (60). The *lpr* murine model of lupus also exhibits a defect in T cell apoptosis due to an impaired Fas signaling pathway (61). This pathway appears to be normal in human SLE and it is unlikely that this specific pathway contributes to disease pathogenesis (62). Of greater relevance is the ability of autoimmune T cells to survive activation induced cell death, which appears to occur in both SLE and murine models. Paradoxically, lupus patients also present with increased spontaneous T cell apoptosis (63). It has been suggested that disruption of the mitochondrial membrane potential at several checkpoints leads to apoptosis in lupus T cells (64). This disease feature may also increase the availability of autoantigen.

Hypo-responsive T cells

T cell responses in SLE are also defective. Peripheral blood mononuclear cells from lupus patients have been shown to be hypo-responsive when stimulated to proliferate in vitro (65). In some patients, T cell proliferation was diminished following treatment with mitogens, anti-CD2, and allogeneic stimulation (66, 67). In another study purified T cells from SLE patients had normal proliferation responses, but deficient cytolytic activity compared with normal controls (68). These findings are paradoxical to the presence of a systemic inflammatory autoimmune response that is, as described above, T cell driven. While reductions in circulating T cells may be explained by the presence of anti-lymphocyte antibodies, it is unclear what causes T cells to be unresponsive in lupus. Further contrasting these defects are reports that suggest abnormal spontaneous proliferation in T cells from lupus patients (69).

T Follicular Helper Cells and IL-21

The production of IL-21 by follicular T helper cells (T_{FH}) provides help to follicular B cells, driving differentiation to antibody secreting plasma cells (70). As such there has been recent interest in its role in lupus. IL-21 plays a critical role in the pathogenesis of lupus like disease in the BXSB-Yaa model of SLE (71). In one model T_{FH} were required for lupus like disease (72). In human studies, two single nucleotide polymorphisms in the IL-21 gene have been associated with lupus (73). Given the potentially pathogenic role of this cytokine, it has been promoted as a potential therapeutic target for autoimmune disease, including lupus (74).

$CD8^+$ T Cells and the IFN Signature

In contrast to the critical role for $CD4^+$ T cells in lupus pathogenesis, CD8 are not thought to play a significant role. While one study found that CD8 T cells isolated from lupus patients were capable of stimulating autoantibody production in B cells in vitro, this report has not been corroborated by others (75). However Type I and Type II interferons, which are important to the cytotoxic activity of $CD8^+$ T cells and to the control of viral infections, have been implicated in lupus (76). Several groups have shown elevation in genes regulated by IFN-α, often referred to as the "IFN signature" (77-79). Plasmacytoid dendritic cells are thought to be the primary source of this cytokine. IFN-α activates immature dendritic cells, which can then enhance the autoimmune response (80). Due to the importance of these cytokines in the control CTL activity it has been posited that viral infection may play a role in initiating lupus. Several reports have linked an increased incidence of EBV infection and viral load with SLE activity (21). Additionally, T cell control of EBV has been shown to be defective in SLE (22). While these phenomena may play a role in the induction of SLE, they may also occur as a result of the immune dysfunction already present in individuals with SLE.

Epidemiology/Risk Factors/Mortality

The reported prevalence of SLE varies between studies. One estimate puts the prevalence in the continental US at 14.6 to 50.8 per 100,000 (81). However other studies report higher rates of 122 and 124 per 100,000 in the US (82, 83). The variability in these studies may be due to differences in data collection, but may also reflect difficulties in

diagnosing SLE in patients. The incidence of SLE in the US is reported at 1.5 to 7.6 per 100,000 annually (84). Changes in ACR classification criteria for SLE may also have contributed to variability in the reported incidence and prevalence in the disease (85).

Risk Factors

Epidemiological studies have demonstrated grouping of SLE patients within families, suggesting that there is a genetic component to the disease (86, 87). Several genetic factors, in fact, have been associated with increased susceptibility to SLE. The DR2 and DR3 alleles of human leukocyte antigen (HLA) Class II have long been associated with SLE susceptibility (88, 89). As mentioned previously, genetic defects such as deficiencies in the complement pathway have been associated with SLE (reviewed in (12)). C1q deficient individuals in particular have a high incidence of lupus.

Evidence indicates that environmental factors may also be a trigger for SLE (90-92). As mentioned previously, it has long been suspected that viral infections may be critical in the pathogenesis of SLE, but no definitive study on this hypothesis has been produced (93, 94). However it is unclear whether increased infection is a cause of SLE or the result of an increased susceptibility to infection in patients with the disease. Exposure to certain chemicals has also been linked to SLE. Occupational exposure to crystalline silica has been shown to increase risk (95). Chemicals that alter sex-hormone homeostasis such as trichlorethyline have also been proposed as potential risk factors (reviewed in (92)). Finally drug induced SLE has been well documented in patients receiving hydralazine, procainamide, isoniazid, TNF-α blockers, and other drug treatments for separate health conditions (96, 97).

Women in childbearing years have an increased risk of SLE, with a female to male ratio of approximately 9 to 1 during this time frame (98, 99). This ratio is not seen in pre-adolescent and post-menopausal females. The time line strongly suggests that sex hormones play a critical role in disease expression. Consistent with this theory, pregnancy has been associated with increased disease activity including flairs (100, 101). Exogenous estrogen treatments have also been linked to increased risk of SLE (102).

Mortality

Survival in SLE patients has improved over the last 50 years. Studies performed in the US from the 1940's to 1960's have reported a 5-year survival rate of less than 50% in patients with SLE (103-105). More recent studies have put the 5 year survival in developed countries at approximately 95% (reviewed in (105)). Standardized mortality ratios (SMR), which compare the observed deaths in a group of interest to the predicted number of deaths in the larger population matched by age, sex, or other parameters, have also improved in that time. One such study performed at the University of Toronto Lupus Clinic showed a reduction in SMR from 12.6 in 1970 to 1979 down to 3.46 in 1996 to 2005 (106). Some of the improvement in survival can be attributed to the wider availability of dialysis treatment for renal failure. The improvement in survival may also be due to earlier detection and treatment of disease, as well as diagnosis of milder cases of SLE. A recent study on lupus mortality reported that diseases of the circulatory system including the heart, infectious disease, and renal failure were more common causes of death in SLE patients than the general population (107). However, circulatory and infectious diseases were more common causes of death than renal failure for lupus

patients. This highlights a shift in the cause of death from renal disease, which can be treated by dialysis, to infectious and circulatory diseases. Increased rates infectious disease may also be attributable to treatment with immunosuppressive drugs in addition to the hypo-responsive T cells seen in lupus.

Murine Models of SLE

Several murine models have been used in the study of lupus. Of them only a few demonstrate a female predominance comparable to human lupus. The MRL/*lpr* model is a spontaneous model of SLE that develops lupus like glomerular nephritis as a result of anti-DNA and anti-nuclear antibody production (108). It does not, however, display a female predominance in disease severity. The BXSB model is a spontaneous model of SLE that displays a male predominance in disease severity, which also does not reflect the human disease (109). The NZB/NZW F1 model is a spontaneous model with a female predominance in disease severity, comparable to human lupus (110). Prior work in this model has made significant discoveries on the role of estrogen in increasing disease severity and the contrasting role of androgens in reducing it. However, it is a spontaneous model making study of the early events in disease pathogenesis difficult. An induced model of murine lupus like disease that also displays a female predominance is the DBA into B6D2F1 (BDF1) model of chronic graft versus host disease (cGVHD)(111). In this model parental strain donor DBA/2J $CD4^+$ T cells are transferred into unirradiated B6D2F1 hosts that are tolerant to parental strain DBA epitopes. While a mild F1 anti-parent response does occur it does not result in graft rejection, thus eliminating the need to irradiate and destroy the host immune system. As such, DBA donor $CD4^+$ T cells enter

a normally functioning host immune system where they recognize and are activated by the C57BL/6 parental strain MHC class II (I-A^b) that is present on all host B cells. Subsequently, they provide cognate T cell help to all host B cells including those capable of responding to autoantigen. This bypasses the requirement for activated autoreactive T cells to provide help for autoreactive B cells. Similar to human lupus, the resulting polyclonal activation of B cells is not random, but rather directed against DNA and other nuclear targets (112).

Sex Based Studies in the DBA into F1 Model

It has previously been demonstrated that the DBA into BDF1 model of cGVHD displays a strong female predilection in disease severity (111). Initial studies showed that multiple transfers of sex matched DBA/2 splenocytes into male and female BDF1 hosts results in a lupus like glomerulonephritis that was more severe in female hosts (111). This was the result of increased production of autoantibodies in female cGVHD mice. Similar to studies in the NZB/W model, this group performed an additional study in which male and female mice were castrated or ovariectomized and treated with either 17β-ethinyloestradiol or testosterone-decanoate following the transfer of DBA/2 splenocytes (113). It was found that ovariectomized females treated with testosterone-decanoate had lower proteinuria levels than sham operated female cGVHD mice, whereas ovariectomized females treated with 17β-ethinyloestradiol did not differ significantly from the sham operated female group. Similar to other studies in murine lupus involving the manipulation of sex hormones, it was thus concluded that androgens had an inhibitory effect on lupus like disease whereas estrogens had a stimulatory effect. These findings

demonstrate the sex-based differences in the DBA→F1 model are comparable to those differences seen in the NZB/W model and in human SLE in being directly influenced by sex hormones. However, similar to previous studies this report did not determine the differences in cellular events that distinguish T cell responses in the male immune system from that in the female immune system.

More recently it has been demonstrated that a single transfer of 80-90 x 10^6 unfractionated DBA/2 splenocytes into age and sex matched BDF1 hosts results in a more severe glomerulonephritis at 10 to 12 weeks post-injection (44). As in previous studies, female cGVHD mice produced higher levels of autoantibodies than males, including antibodies against ssDNA. Females also had increased proteinuria in comparison to male cGVHD mice. Short term studies demonstrated that at two weeks post-injection f→F mice had 2 to 3 fold greater engraftment of pathogenic donor $CD4^+$ T cells than that seen in m→M mice, and that this difference was predictive of long term disease severity. Given the critical role of $CD4^+$ T cells in initiating both murine lupus like disease and human SLE, this study made further efforts to understand the cause of the day 14 differences in donor $CD4^+$ T cell engraftment. It was found that the difference in engraftment was the result of increased donor $CD4^+$ T cell proliferation in females from days 10 to 14 and was not the result of differences in apoptotic rate, proliferation from days 0 to 7, or homing to the spleen following injection.

What remains unclear following these studies is the mechanism behind increased donor $CD4^+$ T cell proliferation in female cGVHD. It was demonstrated that increased donor $CD4^+$ T cell engraftment at week 2 segregates with the sex of the host and not that of the donor. That is, transfers of m→F mice displayed greater engraftment of donor

$CD4^+$ T cells at two weeks than that seen in f→M mice. These findings suggest that there are critical differences between the male and female host immune system that either inhibit or enhance the proliferation of pathogenic donor $CD4^+$ T cells, which subsequently impacts the severity of lupus like disease. The focus for our research then was to investigate differences in $CD4^+$ T cell help to B cells in male and female cGVHD.

In contrast to the critical role for donor $CD4^+$ T cells in inducing lupus like disease, donor $CD8^+$ T cells in the DBA into BDF1 model are not thought to play a significant role. In other p→F1 models, MHC Class I mismatched $CD8^+$ T cells in fact prevent lupus like disease by converting chronic GVHD into acute GVHD. In such cases the host immune system, including B cells, are destroyed by donor $CD8^+$ CTL activity and F1 hosts subsequently exhibit a potentially lethal immune deficiency (114). However, if CTL activity is defective (such is with perforin deficient donor cells) acute GVHD is converted back to lupus like chronic GVHD. The DBA→F1 model is one such exception. As a result of low CTL precursor frequency for F1 MHC alloantigen and defective $CD4^+$ T cell help to $CD8^+$ T cells, the initial graft versus host CTL response is limited and fails to eliminate host B cells completely. Since it is well established that T cell help to B cells is indeed provided by $CD4^+$ and not $CD8^+$ T cells we sought to demonstrate that sex based differences in lupus like disease could occur in the absence of alloantigen specific $CD8^+$ T cells. It was expected that, if the DBA $CD8^+$ T cell had any role in lupus like disease pathogenesis it would be an inhibitory one. Indeed, one study demonstrated that by increasing donor $CD4^+$ T cell help to donor $CD8^+$ T cells by depleting the donor inoculum of $CD4^+Foxp3^+$ regulatory T cells prior to injection, CTL activity was enhanced resulting in acute GVHD (115). While a lupus like disease was

achieved in the absence of donor $CD8^+$ T cells we show here that sex based differences were prevented through reduced disease severity in CD8 depleted f→F mice and a worsening in CD8 depleted m→M mice.

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Chapter 2

Donor CD8 T cell activation is critical for sex based differences in lupus-like disease in chronic GVHD mice: I. A role for ICOS^{hi} host CD4 T cells and IL-21 in promoting greater renal disease severity in females[1].

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Keywords: graft-vs-host disease, Fas, costimulation, lupus

Abstract

The incidence of human lupus and accompanying glomerulonephritis (GN) is largely a female disease. To address sex based differences in lupus renal disease expression, we used the DBA-into-F1 murine model of lupus-like renal disease in which disease is more severe in females. In this model, disease is induced by the transfer of unfractionated DBA/2 donor splenocytes. CD4 T cells within the donor inoculum provide cognate help to semi-allogeneic host B cells. Donor CD8 T cells in the donor inoculum are not thought to be required. To directly confirm the central role of DBA/2 CD4 T cells, we transferred DBA/2 splenocytes depleted of donor CD8 T cells and observed that, as expected, lupus-like disease ensued. Surprisingly, sex based differences were lost. Specifically, using unfractionated DBA splenocytes (CD8 intact→F1) f→F1 mice exhibited clinical and serological evidence of nephrotic syndrome, greater histological severity of GN and greater glomerular deposition of Ig compared to m→M mice. Electron microscopy confirmed that f→F mice exhibited a severe membranous GN vs. m→M mice. These sex based differences in GN severity were lost in CD8 depleted→F1 mice due to an improvement in f→F disease and a worsening of m→M disease. Flow cytometric analysis of splenic T cells at 13 weeks of disease indicated that CD8 intact f→F mice exhibited greater donor and host effector (CD44hi, CD62Llo) CD4 T cells and greater $ICOS^{hi}$ CD4 T follicular helpers. CD8 depleted→F1 mice continued to exhibit sex based changes in donor CD4 T cells however sex based changes in host CD4 effectors were lost and both host CD4 effector and Tfh subsets were significantly reduced. IL-21 gene expression, a product of Tfh, was also greater in CD8 intact→F1 female vs male mice and this difference was reduced for CD8 depleted →F1 mice. These

results support a role for host CD4 T cells, particularly the $ICOS^{hi}$ Tfh cell subset, in mediating sex based differences in lupus GN severity, possibly through IL-21 production.

Note: M. Haas performed all histological analysis in this study. Technical assistance on some experiments was provided by R. Puliaev, I. Puliaeva, or K. Soloviova.

Introduction

A hallmark of systemic lupus erythematosus is pathogenic autoantibody formation directed against nuclear antigens, particularly chromatin (1). Although B cells are intrinsically abnormal in some murine models of lupus (2), pathogenic IgG autoantibodies in SLE typically exhibit features characteristic of a normal antigen driven response e.g., somatic mutation, affinity maturation and T cell help (3-7). CD4+ T cells are central in driving B cell autoantibody production in both human and murine lupus (8-10) and the autoantigens recognized by both T and B cells are derived from chromatin and ribonucleoproteins (8-16). Although CD8 T cells have been described that are capable of supporting antibody production by lupus B cells in vitro (17) such CD8 Th cells remain an uncommon finding compared to the large body of evidence demonstrating that CD4 T cells are necessary and sufficient for lupus development.

A useful model for studying the mechanistic role of CD4 T cells in lupus pathogenesis is the parent-into-F1 (P→F1) model of chronic graft-vs-host disease (GVHD). In this model, a lupus-like disease is induced in normal F1 mice following the transfer of parental strain CD4+ T cells (18). Disease is a consequence of donor CD4 T cell recognition of allogeneic host MHC II molecules (19, 20) resulting in cognate delivery of CD4 help to all host B cells (21) that in turn results in polyclonal host B cell activation, the production of characteristic lupus-related autoantibodies and an immune complex glomerulonephritis (22-24). Because mouse T cells do not express MHC II, lupus like cGVHD can be induced in P→P transfers that involve solely an MHC II disparity e.g., B6→bm12 or vice versa (25). Importantly, if parental CD8 T cells are administered in addition to CD4 T cells and the disparity between parent and F1 involves both MHC I and II, disease phenotype is converted from a lupus like chronic GVHD to an acute lethal

disease in which donor CD8 T cells mature into CTL specific for host MHC I. The resulting attack on host immune and hematopoetic system results in a profound immunodeficiency that can be lethal. Thus, lupus like disease in this model is thought to result solely from the activation of an oligoclonal population of alloreactive donor CD4 T cells that are specific for host MHC II and that concurrent activation of donor CD8 T cells qualitatively alters disease phenotype such that B cells are eliminated and lupus like disease is prevented.

Lupus-like auto antibodies are seen in a number of different of P→F1 combinations (26) however only a few combinations result in sustained lupus-like immune complex glomerulonephritis (ICGN) that resembles human lupus renal disease. One of the best studied is the transfer of DBA/2 lymphocytes into B6D2F1 mice (DBA→BDF1) (22, 23, 27-29). This p→F1 combination has been shown to exhibit sex based differences with greater disease severity of renal disease seen in females (30-32)**.** It should be noted that lupus-ike disease in DBA→F1 mice is seen following the transfer of both CD4 and CD8 T cells into a fully allogeneic (MHC I + II disparate) B6D2 F1 and is an exception to the rule that the co transfer of both CD4 and CD8 T cells results in acute GVHD. Although an acute GVHD phenotype is the expected outcome in DBA→F1 mice, maturation of donor DBA CD8 T cells into anti-host effector CTL is defective due in part to an approximate 10-fold reduction in the anti-F1 pCTL frequency compared to B6 mice (33) and preferential Th2 cytokine production by DBA CD4 T cells (34). As a result, DBA→F1 mice develop chronic lupus like GVHD rather than acute GVHD. Donor DBA CD8 T cells have not been shown to play a role in lupus-like disease expression much less in sex based differences in disease severity in DBA→F1 mice,

In this study, we initially sought to examine sex based differences in donor CD4 Th cell function for B cells by formally demonstrating that donor CD8 T cells are irrelevant to disease expression. Surprisingly, we observed that DBA CD8 T cells play a

critical role by exerting differential effects on host CD4 T cells, particularly $ICOS^+$ cells, and on IL-21 production.

Materials and Methods

Mice: 6-8 week old male and female DBA/2J (DBA) (H-2^d) and B6D2F1 (BDF1)(H-$2^{b/d}$) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). All animal procedures were pre-approved by the Institutional Animal Care and Use Committee at the Uniformed Services University of Health Sciences.

Induction of GVHD: Single cell suspensions of DBA splenocytes were prepared as described (35) and transferred into BDF1 hosts by tail vein injection. Donor and host mice were age and sex matched such that male donors were transferred into male hosts (m→M) and female donors into female hosts (f→F). Donor splenocytes were first analyzed by flow cytometry for CD4 and CD8 T cells and F1 mice received either: a) unfractionated splenocytes (CD8 intact→F1) containing 14 x 10^6 CD4 T cells and 4.6 x 10^6 CD8 T cells (approximately 80-90 x 10^6 total splenocytes); or b) splenocytes depleted of CD8 T cells (CD8 depleted→F1) containing 14 x 10^6 CD4 T cells. For CD8 depletion studies, CD8 T cells were positively selected and removed from the donor population using magnetic beads purchased from Invitrogen (Carlsbad, CA) according to the manufacturer's instructions. Flow cytometry analysis before cell transfer confirmed <1% contaminating $CD8^+$ T cells.

Flow cytometric analysis. Spleen cells were first incubated with anti-murine Fcγ receptor II/III mAb, 2.4G2 for 10 min and then stained with saturating concentrations of Alexa Fluor 488-conjugated, biotin-conjugated, PE-conjugated, APC-conjugated, PerCPCy5.5-conjugated, Pacific Blue-conjugated, and Pacific Orange-conjugated mAb against CD4, CD8, B220, H-$2K^b$, I-A^b, CD44, CD62L, ICOS (CD278), CD80, CD86, CD21, CD23, and CD138 were purchased from

either BD Biosciences (San Jose, CA), BioLegend (San Diego, CA), eBiosicnce (San Diego, CA), or Invitrogen (Carlsbad, CA). Biotinylated primary mAb were detected using either streptavidin-PE-Texas Red (BD Bioscience) or streptavidin-Alexa Fluor 700 (Invitrogen). Cells were fixed in 1% paraformaldehyde. Multi-color flow cytometric analyses were performed using a BD FACScan flow cytometer or BD LCRII flow cytometer (BD Biosciences, San Jose, CA). Lymphocytes were gated by forward and side scatter and fluorescence data were collected for a minimum of 10,000 gated cells. Studies of donor T cells were performed on a minimum of 4,000 gated cells that were positive for $CD4^+$ or $CD8^+$ and negative for MHC class I of the uninjected parent.

Cytokine Expression by PCR: RNase-free plastic and water were used throughout the assay. Splenocytes (1×10^7) were homogenized in 1 ml of RNA-STAT-60 (Tel-Test, Friendswood, TX). RNA samples were reverse transcribed with Moloney murine leukemia virus reverse transcriptase (Invitrogen, Carlsbad, CA). Primers and probes were purchased from Applied Biosystems International (ABI). All real time PCR primers were purchased from Applied Biosystems.

Kidney Histopathology. Kidney tissue was fixed in 10% buffered formalin and processed for routine paraffin embedding and histological sectioning. Three-micron-thick sections, stained with periodic acid-Schiff stains, were blindly scored by a renal pathologist (MH). The severity of proliferative glomerulonephritis (GN score) and interstitial nephritis (IN score) were determined according to a previously described semiquantitative scoring system developed for a murine model of lupus nephritis (36).

Immunofluorescence Microscopy. Two micron-thick cryostat sections of unfixed kidney tissue were cut, allowed to air dry, and were fixed in cold acetone for 10 minutes. After rinsing twice with cold PBS, the sections were incubated for 30 minutes at room temperature in a humidified chamber with fluorescein isothiocynate conjugated polyclonal antisera against mouse IgG1 (1:10 dilution in PBS) or IgG2a (1:20 dilution) (both from Southern Biotech, Birmingham, AL). Slides were then rinsed twice for 5 min each in PBS and coverslipped with Fluoromount. Immunofluorescence staining intensity in glomerular capillary walls and mesangium were blindly scored for each slide by a renal pathologist (MH), using a semiquantitative scale of 0 – 4, in increments of 0.5

Electron Microscopy. Ultrastructural features were examined in glomeruli from 1-2 mice from each of the experimental groups. Formalin-fixed renal cortex was post-fixed in 3% glutaraldehyde and processed for electron microscopy using standard techniques as described previously (37). Ultrathin sections were viewed with a Philips CM10 electron microscope (Bothell, WA), and representative photographs were taken.

Urine Protein. Urine protein was quantitated by dipstick (Albustix, Bayer Pittsburgh, PA).

Serum chemistry. Serum was obtained at 13 weeks at the time of sacrifice and serum BUN, cholesterol, albumin and triglycerides were determined using a VITROS 350 Chemistry System (Ortho-Clinical Diagnostics, Rochester, NY) according to the

manufacturer's protocol.

Serological studies. Mice were bled at the times indicated and sera tested by ELISA for the presence of IgG antibodies to ssDNA as described (38).

Statistical Analysis. Data was analyzed by unpaired Student's t test. Non parametric data were analyzed by Mann-Whitney.

Results

Donor CD8 T cells are required for sex based differences in severity of lupus-like renal disease in DBA→F1 mice. We first sought to determine whether sex based differences existed in the ability of DBA CD4 T cells to provide help to F1 B cells. In these experiments we eliminated a contributory role for donor CD8 T cells by transferring DBA donor splenocytes depleted of CD8 T cells into a cohort composed of both m→M and f→F transfers (CD8 depleted→F1) and uninjected age and sex matched control F1 mice. A second control cohort consisted of age matched m→M and f→F transfers receiving unfractionated DBA splenocytes (CD8 intact→F1) and uninjected age and sex matched F1 mice. All donor inocula contained 14 x 10^6 CD4 T cells and the CD8 intact cohort also received 4.6 x 10^6 CD8 T cells. By 13 weeks, clinical evidence of nephrotic syndrome was observed only in members of the CD8 intact f→F group and included ascites (3/5 mice) and grossly lipemic serum (5/5 mice). Ascites and grossly lipemic serum were not observed in any of the other three experimental groups.

CD8 intact f→F mice also exhibited significantly greater BUN (Fig. 1A), lower albumin (Fig. 1B) and greater triglycerides (Fig. 1C) compared to CD8 intact m→M. Both groups exhibited elevated cholesterol compared to uninjected control mice (Fig. 1D). Importantly, most of these sex based differences were lost in CD8 depleted →F1 mice. Specifically, there were no significant decrease in serum albumin and no significant differences in cholesterol or triglycerides between m→M and f→F GVHD mice receiving CD8 depleted donor cells. However, f→F mice still exhibited significantly greater serum BUN and greater serum albumin compared to m→M GVHD mice.

Sex based differences in renal disease severity are lost with donor CD8 T cell depletion. Both cohorts were sacrificed at 13 weeks and kidneys scored blindly for glomerular and tubulo-interstitial disease severity as described in Methods. For the CD8 intact→F1 cohort, sex based differences were seen for both glomerular (Fig. 2A) and interstitial scores (Fig. 2B) with f→F exhibiting significantly higher scores than m→M GVHD mice. Only 1/5 m→M mice exhibited glomerular disease (Glomerular score >1+) vs. 5/5 f→F GVHD mice. Sex based differences in renal scores were lost in DBA→F1 mice receiving CD8 depleted donor splenocytes due to a combination of worsening of some m→M scores and improvement in some f→F scores.

CD8 intact f→F GVHD mice exhibit severe membranous GN. Nephrotic syndrome may result from underlying membranous GN. To examine the pathologic basis of the clinical and serum chemistry results indicating severe nephrotic syndrome in CD8 intact f→F GVHD mice, immunofluorescent staining for deposited IgG1 and IgG2a was performed on tissue from both CD8 intact→F1 and CD8 depleted→F1 cohorts and severity of deposition quantitated as described in Methods and shown in Fig. 2 C-F. Sex based differences are seen for CD8 intact→F1 mice but not for CD8 depleted→F1 mice. Specifically, CD8 intact f→F mice exhibited significantly greater glomerular capillary wall deposition of both IgG2a (Fig. 2C) and IgG1 (Fig. 2D) whereas no significant sex-based differences were seen for CD8 depleted→F1 mice. By contrast, reverse sex based differences were seen for mesangial deposition with CD8 intact m→M mice exhibiting

significantly greater mesangial deposition of IgG2a compared to CD8 intact f→F mice (Fig. 2E). There were no significant differences in mesangial IgG1 deposition between these two groups (Fig. 2F). CD8 depleted→F1 mice did not exhibit sex based differences in IgG1 or IgG2a deposition in either the GCW or the mesangium. As with the renal scores in Figs. 2A, B, the loss of sex based differences in CD8 depleted→F1 mice results from worsening in some m→M mice and improvement in some f→F mice.

Representative IF staining for IgG2a is shown in Fig. 3 and demonstrates no detectable deposition in either uninjected male (Fig. 3A) or female (Fig. 3B) control F1 mice. CD8 intact m→M mice exhibit a predominantly mesangial pattern with weak GCW staining (Fig. 3C) whereas CD8 intact f→F mice exhibit striking, confluent granular GCW staining in a typical membranous pattern, in most instances without mesangial staining (Fig. 3D). CD8 depleted m→M (Fig. 3E) and f→F mice (Fig. 3F) exhibit a mixture of mesangial and GCW staining, the latter being milder than in CD8 intact f→F mice.

Electron microscopy confirms the membranous pattern and also demonstrates more extensive membranous change in CD8 intact f→F mice (Fig.4). A normal male F1 (Fig. 4A) demonstrates only very rare mesangial electron-dense deposits (EDD) (arrow). No subepithelial deposits are present and podocyte foot processes are generally intact. CD8 intact m→M (Fig. 4B) demonstrates expansion of mesangial matrix with numerous mesangial EDD consistent with the immunofluorescence findings for IgG2a deposition (Fig. 2E and Fig. 3). There are multiple subepithelial deposits, although in a somewhat segmental distribution with absence of deposits in some portions of capillary loops. Very rare subendothelial deposits were also noted (arrow), and there is extensive, almost

complete podocyte foot process effacement. By contrast, CD8 intact f→F (Fig. 4C) shows numerous subepithelial deposits diffusely involving capillary loops, as well as a small number of mesangial deposits (arrow). Extensive, almost complete podocyte foot process effacement is also seen. Figs 4D & E are from mice receiving CD8 depleted donor inocula and demonstrate for m→M (Fig. 4D) an expansion of mesangial areas with a smaller amount of mesangial EDD (arrows) than in CD8 intact m→M, again consistent with the immunofluorescence findings for IgG2a in this group (Fig. 2E). There are also many subepithelial deposits, although as with CD8 intact m→M there was an absence of deposits in some portions of capillary loops (arrowhead). Podocyte foot processes are diffusely effaced. Similarly, for CD8 depleted f→F (Fig. 4E) there is also mild expansion of the mesangial matrix with occasional mesangial EDD (arrow). There are numerous subepithelial deposits, albeit not quite as confluent as in CD8 intact f→F (Fig. 4C). Podocyte foot processes are diffusely effaced.

Taken together, the results of Figs 2 – 4 support the conclusion that all DBA→F1 mice exhibit membranous GN, but that the amounts of subepithelial and mesangial immune complex deposit are altered by the sex of the P→F1 combinations and the presence or absence of CD8 T cells in the donor inoculum with the greatest severity seen in CD8 intact f→F mice.

More severe renal disease in CD8 intact f→F GVHD mice is preceded by greater proteinuria and serum anti-ssDNA ab. For CD8 intact→F1 mice, both m→M and f→F mice shown a similar initial mild increase in proteinuria peaking during weeks 2- 4 after which the two groups diverge with proteinuria increasing from weeks 6-10 for CD8

intact f→F mice as opposed to a decline in CD8 intact m→M mice. The differences in proteinuria become significant at weeks 8 and 10. Sex based differences are not seen in CD8 depleted→F1 mice with both m→M and f→F mice exhibiting similar severity levels and both groups exhibit a similar peak at 8 weeks.

Anti-ssDNA ab are a useful marker of autoreactive B cell activation in this model (38) and confirm the foregoing results demonstrating sex based differences in proteinuria in CD8 intact DBA→F1 mice that are lost in CD8 depleted DBA→F1. As shown in Fig. 5A, CD8 intact→F1 f→F GVHD mice exhibit striking increase in anti-ssDNA ab peaking at 6 weeks significantly higher than that of CD8 intact m→M GVHD mice (f→F vs. m→ M = 3-fold at 6 weeks). These sex based differences are not seen for CD8 depleted→F1 mice due to a striking reduction in f→F anti-ssDNA values rather than an elevation of m→M values.

Sex based differences in F1 B cell numbers and activation are seen in the CD8 intact -->F1 cohort and lost in the CD8 depleted→F1 cohort. To determine whether the greater autoantibody production in CD8 intact f→F vs. m→M mice was accompanied by changes in T cell and B cell parameters, the spleens of both cohorts were examined at 13 weeks at the time of sacrifice for lymphocyte populations. As shown in Fig. 6, sex based differences were observed in the CD8 intact cohort with f→F mice exhibiting significantly greater numbers of total B cells (Fig. 6A), follicular B cells (6B), plasma cells (6C) and significantly greater upregulation of MHC II on B cells (Fig. 6D) compared to m→M mice. These sex based differences are lost in the CD8 depleted→F1

cohort due to a combination of a reduction in f→F values and an increase in m→M values. Nevertheless, B cell parameters in Fig. 6 remain elevated over control F1 for both sexes in the CD8 depleted→F1 mice consistent with active renal disease shown in Figs. 2-4.

Donor CD8 T cells are associated with alterations in host CD4 Th subsets, particularly T follicular helper (Tfh) cells. CD4 T cells play a critical role in promoting B cell autoantibody production in both human lupus (1, 10, 39) and the p→F model of lupus (20, 21). CD4 Th cells can be divided into naïve and activated subsets based on their expression of CD44 (hi and low respectively) and CD44hi CD4 T cells further subdivided with combined CD62L expression such that CD44hi, CD62Llow are composed of activated effector cells and CD44hi, CD62hi are composed of central memory cells (40)**.** Importantly, upregulation of ICOS designates the T-follicular helper (Tfh) subset shown to be critical in providing help to B cells and promoting Ig production in large part through IL-21 (41-43). The numbers of these CD4 T cell subsets are shown in Fig. 7 and demonstrate sex based differences for the CD8 intact→F1 cohort for both donor (Fig. 7A, B) and host (Fig. 7C,D) CD4 T cell subsets. For example, CD8 intact f→F mice exhibit significantly greater numbers of donor T fh (Fig. 7A), total donor CD44hi CD4 T cells (Fig. 7A), donor effector CD4 T cells (CD44hi, CD62Llo)(Fig. 7B) and donor central memory CD4 T cells (CD44hi, CD62Lhi)(Fig. 7B) compared to CD8 intact m→M mice. Regarding host CD4 T cell subsets, similar sex based differences are observed i.e. CD8 intact f→F mice exhibit significantly greater numbers of host T follicular helper (Fig. 7C), total host CD44hi (Fig. 7C), host effector helper (CD44hi,

CD62Llo)(Fig. 7D) and host central memory CD4 T cells (CD44hi, CD62Lhi)(Fig. 7D). Importantly, donor CD8 depletion had differing effects on donor vs host CD4 subsets. Specifically, sex based differences in donor CD4 subsets are preserved in the CD8 depleted cohort and the numbers of CD4 Th cell subsets for f→F mice do not differ significantly comparing CD8 intact to CD8 depleted groups. Although donor Tfh cells in f→F mice exhibited a trend towards reduction in CD8 depleted cohort compared to CD8 intact cohort this difference was not significant**.**

In contrast to donor CD4 T cells, some sex based differences in host CD4 T cells are lost in the CD8 depleted cohort. Notably, the CD8 depleted cohort exhibits significant reductions in host CD4 Tfh cells for both m→M and f→F GVHD mice compared to their respective groups in the CD8 intact cohort. Despite these overall reductions, Tfh cells remain significantly greater in f→F mice vs m→M mice in the CD8 depleted cohort. Additionally, sex based differences in host CD4 effector cells were lost in the CD8 depleted cohort. CD8 depleted f→F mice also exhibited reductions in CD44hi and CD4 effector populations compared to CD8 intact f→F mice however these differences were not significant. Thus, the major effect of donor CD8 T cell depletion is a reduction in the absolute numbers of host, not donor, CD4 Tfh cells and a loss of female skewing of CD4 effector (CD44hi, CD62Llow) cells.

Sex based differences in IL-21 gene expression are seen in CD8 intact →F1 cohort and lost in the CD8 depleted →F1 cohort. ICOS-dependent Tfh cells play an important role in promoting B cell maturation and IgG production in part through production of IL-

21 (41, 44, 45). Importantly, IL-21 has been also shown to be important in the production of IgG autoantibodies in spontaneous murine lupus (46)**.** To further address a possible role for Tfh cells in the DBA→F1 model of lupus, we sought evidence for sex based differences in IL-21 by quantitating IL-21 gene expression from F1 spleens at 13 weeks. For CD8 intact→F1 mice, f→F mice exhibit striking elevations of IL-21 gene expression over sex matched control F1 mice (Fig. 8A). Moreover, these values are approximately 4 fold greater than those seen in m→M CD8 intact mice. By contrast, in the CD8 depleted cohort, IL-21 gene expression is significantly reduced for f→F mice compared to f→F mice in the CD8 intact cohort however it is still significantly greater than that for m→M mice. No significant sex based differences were seen for IFNa inducible gene mx-1 (B), OAS (data not shown) Interestingly, there was significantly greater IFN-g (Fig. 8C) and IL-6 (Fig. 8D) gene expression for CD8 intact m→M vs f→F mice and these sex based differences were lost in the CD8 depleted cohort. These results demonstrate that pattern of IL-21 gene expression demonstrating elevations and sex based skewing with or without donor CD4 T cells is not seen for other cytokines important in T and B cell responses, thereby underscoring the importance of IL-21 in disease expression.

Discussion

Our results make two novel observations regarding the pathogenesis of murine lupus-like renal disease. Firstly, sex based differences (i.e. greater female severity) in clinical and histological severity of GN are critically dependent on the presence of CD8 T cells in the donor inoculum. Secondly, changes in host CD4 T cells, particularly the $ICOS^{hi}$ Tfh cell subset, are associated with sex based differences in disease severity, possibly through IL-21 production. The critical role of donor CD8 T cells was directly demonstrated by depletion of donor CD8 T cells which resulted in the loss of sex based differences in renal disease severity. Because donor CD4 T cells are central to disease pathogenesis in this model, selective depletion of donor CD8 T cells only does not block donor CD4 T cell driven lupus-like renal disease development but instead attenuates the female skewing of severity. The role of host CD4 Tfh cells was indirectly shown by flow cytometry studies demonstrating this subset was the only donor or host CD4 T cell subset that was significantly reduced long term by depletion of donor CD8 T cells. Further supporting a role for host CD4 T cells was a loss of sex based differences in host CD4 effectors seen for CD8 depleted→F1 mice. That is, the greater severity in renal disease for females seen in CD8 depleted →F1 mice is accompanied by greater numbers of donor and host CD4 effector and CD4 $ICOS^{hi}$ Tfh cells in females. Sex based differences in renal disease severity are lost in CD8 depleted→F1 mice and this is accompanied by changes in the host CD4 T cell population and not the donor. Specifically CD8

depleted→F1 mice exhibit a significant reduction in both CD4 effector and Tfh cells vs. CD8 intact→F1 mice and a loss of sex based differences in host CD4 effector cells.

Supporting evidence for a role for Tfh cells was provided by splenic IL-21 gene expression, a product of Tfh cells that promotes IgG antibodies to include autoantibodies. IL-21 gene expression was strikingly increased in f→F compared to m→M mice for the CD8 intact→F1 cohort and these sex based differences were significantly reduced but not totally eliminated in the CD8 depleted→F1 cohort.

To understand how these novel observations advance our understanding of sex based differences in lupus, it is useful to review the currently accepted roles of donor CD4 and CD8 T cells in the pathogenesis of lupus like disease in the p→F1 model. It is well established that donor CD4 T cells are necessary and sufficient for lupus-like disease induction (19, 21, 25)**.** Donor CD4 T cells provide cognate help to host B cells through recognition of allogeneic MHC II molecules resulting in polyclonal B cell hyperactivity, autoantibody production and lupus like renal disease. Moreover, donor T cells exhibit continued helper activity as long as 10 weeks of disease consistent with the conclusion that disease results from sustained CD4 activity rather than a single transient episode at the time of transfer (47)..

By contrast**,** donor CD8 T cells are not required for lupus like chronic GVHD and instead, their inclusion in the donor inoculum typically converts lupus like chronic GVHD to acute GVHD (33). Donor CD8 T cells have a well studied role in mediating acute GVHD following their maturation into effector CTL specific for host MHC I resulting in elimination of host lymphocytes, particularly B cells (reviewed in (48). By

approximately day 12-14 after transfer, donor CD8 CTL undergo homeostatic contraction whereas a similar degree of contraction is not seen for donor CD4 T cells (38). Thus, incomplete elimination of host splenocytes at the time of donor CD8 contraction, as seen following the transfer of donor cells with defective CD8 CTL maturation (e.g. perforin defective donor CD8 T cells) allows re-expansion of host cells, particularly B cells due to the continued presence of donor CD4 T cells capable of providing continued T-B collaboration and the transition to a lupus-like phenotype (49). DBA CD8 T cells exhibit defective in vivo anti-host CTL maturation due to a reduced precursor CTL frequency for F1 MHC alloantigens (33) and a Th2 skewing of DBA CD4 cells (34). As a result, in vivo CD8 CTL development is markedly attenuated in DBA→F1 mice, elimination of host B cells is incomplete and mice develop lupus rather than acute GVHD as donor CD8 T cells contract. Our results indicate that despite the failure of DBA CD8 T cells to mature into CTL effectors capable of completely eliminating host B cells, their sub-optimal activation and downregulation during the first two weeks after transfer is necessary for sex based differences to be seen in this model. We have recently observed that the co-injection of CD8 donor T cells with donor CD4 T cells (i.e. CD8 intact→F1) results in greater donor CD4 proliferation for both sexes than when donor cells are depleted (CD8 depleted →F1) indicating a role for donor CD8 T cells in promoting donor CD4 expansion separate from their role as anti-host CTL (manuscript in preparation). It is possible that greater donor CD8 T cell survival in females promotes greater donor CD4 T cell engraftment that in turn results in more severe disease. This question is currently under investigation in our laboratory.

Regardless of the mechanisms involved in mediating sex based differences in donor CD4 T cell engraftment, our results demonstrate that these initial changes are associated with long term sequellae best seen in the host CD4 T cell compartment. Recent evidence indicates that an ICOS dependent CD4 T cell subset, Tfh cells, provide help for both B cell maturation and IgG production within the germinal cell, in part by production of Il-21 (41-46) a T cell produced cytokine essential for B cell activation, expansion and differentiation into plasma cells (42). Importantly, ICOS dependent Tfh cells have been shown to be critical in the production of lupus-like autoantibodies in two different murine models (41, 50). ICOS positive CD4 T cells and IL-21 gene expression are therefore a relevant measure of CD4 help for autoantibody responses. Our results surprisingly support a role for host rather than donor $ICOS^{hi}$ CD4 T cells in the generation of sex based differences in DBA→F1 mice. Despite the well recognized role for donor CD4 T cells in promoting lupus like disease, we observed a possible important role for host CD4 T cells. Specifically, we demonstrated that the sex based differences in renal disease seen in CD8 intact→F1 mice were accompanied by parallel sex based differences in $ICOS^{hi}$ donor and host CD4 T cell numbers and IL-21 gene expression. However, the loss of sex based differences in renal disease severity seen in CD8 depleted→F1 mice was accompanied by a loss of sex based differences in IL-21 gene expression and in host but not donor $ICOS^{hi}$ CD4 T cells.

These results raise the possibility that in addition to the obligatory role of donor CD4 T cells in lupus induction, host CD4 T cells may play a non-obligatory role that shapes disease expression and severity. A contributory role for host CD4 T cells in the P→F1 model has been long suspected but technically difficult to mechanistically address

separate from donor T cells as B cells do not develop normally in CD4 deficient mice thus precluding the use of such mice as recipients (51). Our results providing associative evidence linking the numbers of host $ICOS^{hi}$ CD4 T cells to sex based differences in disease severity support further studies into the role of host separate from donor CD4 Tfh cells in mediating sex based differences in disease severity. Such studies have important ramifications regarding lupus pathogenesis both in mice and in humans. A longstanding unresolved question in lupus pathogenesis is the mechanism by which T cell tolerance is lost and autoantigens targeted. Moreover, it is not clear whether the ag recognized by T cells that initiate lupus are the same ag recognized by the T cells that perpetuate lupus. An advantage of the p→F1 model is the ability to study the T cells that initiate disease (donor T cells) separate from secondarily expanded and recruited (host) T cells. In the p→F1 model, donor T cells are analogous to the pathogenic T cells that initiate initiate lupus whereas host T cells represent the remaining normal repertoire of non autoimmune prone mice. The p→F 1 model clearly demonstrates that lupus can develop as a result of a normal T cell response abnormally targeted to self MHC II. Although the donor CD4 T cells that initiate disease are normal MHC II alloreactive T cells, the target is self MHC II of the host. Presumably such T cells are deleted in the thymus in normals and this artificial experimental breach of tolerance demonstrates the consequences of such a breach i.e it is not necessary to transfer autoantigen specific CD4 T cells (i.e. anti-chromatin, anti-ssDNA), but rather T cells that will provide cognate help to all B cells. Participation of host CD4 T cells in disease expression supports the idea that once tolerance is broken, the pool of ag-specific CD4 T cells can be expanded, possibly

through epitope spreading and clonal expansion as described (52) to exacerbate disease expression and the production of pathogenic autoantibodies.

Acknowledgements

The authors thank Lee Lewis, USUHS for performing the serum chemistry analysis.

Footnotes

[1] This work was supported by the following grants (to CSV): National Institutes of Health AI047466 and VA Merit Review. Dr. Puliaev is a recipient of an Engelticheff Fellowship.

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[3] Abbreviations used: GVHD, graft versus host disease; P→F1, parent-into-F1;

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Figure Legends

Figure 1. Greater female severity of serum chemistries indicative of nephrotic syndrome seen using CD8 intact donor cells is lost when donor CD8 T cells are depleted. BDF1 mice received undepleted DBA splenocytes or DBA splenocytes selectively depleted of CD8 T cells (<1%). Both cohorts were followed for 13 weeks at which time sera was analyzed for serum A) BUN, B) Albumin, C) cholesterol, and D) triglycerides as described in Methods. Values represent individual mice tested separately. For all figures: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$

Figure 2. CD8 intact f→F mice exhibit more severe renal disease by both histological scoring and Ig deposition. or) Increased severity in renal disease in CD8 intact f→F mice as demonstrated by both histological scoring and Ig deposition is lost in CD8 depleted f→F mice. Kidneys from both cohorts were processed at 13 weeks as described in Methods and scored blindly for A) proliferative glomerulonephritis (GN score), B) interstitial nephritis (IN score), glomerular capillary wall deposition of C) IgG2a or D) IgG1 and mesangial deposition of E) IgG2a or F) IgG1. Immunofluorescence staining intensity in both glomerular capillary walls and mesangium was graded on a 0-4 scale, in increments of 0.5. Values represent individual mice.

FIgure 3. Representative immunofluorescence micrographs of glomerular deposition of IgG2a. Experimental protocol is as outlined in Figs. 1 and 2 and IF staining as described in methods and shown for IgG2a for: A) normal male; B) normal female; C) CD8 intact m→M; D) CD8 intact f→F; E) CD8 depleted m→M and G) CD8 depleted f→F. Normal mice showed no

more than trace (0.5 intensity score) mesangial staining for IgG2a, with no capillary wall staining. Patterns of staining in each of the experimental groups are discussed in the text. Original magnification of each photomicrograph is x400.

Figure 4. Electron microscopy of representative glomeruli from normal and experimental mice. Experimental groups are as described in Fig. 1 and EMs were performed as described in Methods. A) normal male F1; arrow denotes rare mesangial deposits. (original magnification x6300). B) Intact m→M, showing numerous mesangial deposits and multiple subepithelial deposits although in a somewhat segmental distribution with absence of deposit in small portions of the capillary loop. Very rare subendothelial deposits were noted (arrow) (original magnification x8000). C) Intact f→F, showing diffuse, confluent subepithelial deposits, and occasional mesangial deposits (arrow) (original magnification x6300. D) CD8 depleted m→M; there are a smaller number of mesangial deposits than in intact m→M, and multiple subepithelial deposits though with absence of deposit segmentally along the capillary loop (arrowhead) (original magnification x6300). E) CD8 depleted f→F; findings are similar to those in CD8 intact f→F with occasional mesangial deposits (arrow) and many subepithelial deposits, although the latter are somewhat less confluent than in intact f→F (original magnification x6300).

Figure 5. Greater severity of proteinuria and serum anti-ssDNA levels seen for CD8 intact f→F vs m→M mice is lost with donor cell CD8 depletion. Experimental groups are as in Fig. 1. Mice were tested at the indicated time points for proteinuria (A, B) and serum anti-ssDNA ab (C,D) as described in Methods for CD8 intact (A, C) and CD8 depleted (B, D) cohorts. Values represent group mean ± SEM.

FIgure 6. CD8 intact f→F mice have increased numbers of B cells as well as increased B cell activity which is lost with donor cell CD8 depletion. Experimental groups are as in Fig. 1. Total splenocytes were stained for analysis by flow cytometry as described in Methods for A) total B Cells ($B220^{+}$), B) follicular B Cells ($B220^{+}CD21^{Int}CD23^{Hi}$), C) and plasma cells ($B220^{+}CD128^{Hi}$). D) MHC II expression is shown on total B cells and expressed as percent increase in mean fluorescence intensity relative to sex matched F1 control.

Figure 7. Donor CD8 depletion results in a quantitative reduction in host but not donor T follicular helper cells in f→F and m→M mice. Experimental groups are as in Fig. 1. Total splenocytes were stained for analysis by flow cytometry as described in Methods for: A) total donor CD4 T cells and total donor CD44hi T cells, B) total donor CD44hi,CD62Llo and total CD44hi, CD62Lhi T cells, C) total host CD4 T cells and total host CD44hi T cells, and D) total host CD44hi,CD62Llo and host CD44hi, CD62Lhi T cells,

Figure 8. CD8 intact f→F mice have greater expression of IL-21 than m→M mice and this difference is reduced in CD8 depleted→ F1 mice. Experimental protocol is as in Fig. 1. Splenic mRNA was isolated and cytokine gene expression performed as described in Methods. Results are shown as group mean of **individual values** ± SEM for A) IL-21, B) Imx-1C) IFN-g and D) IL-6.

Fig. 1

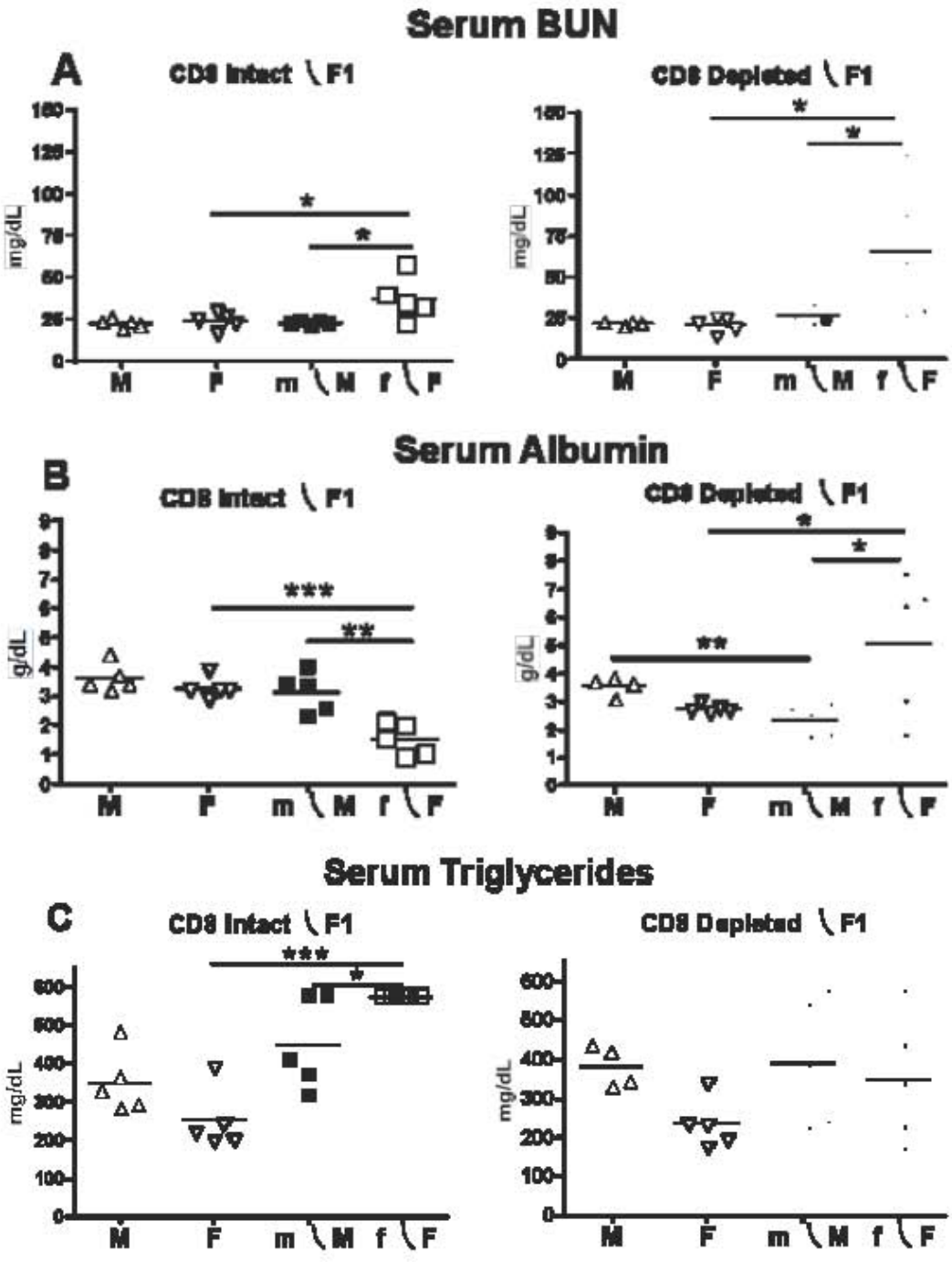
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Fig. 1

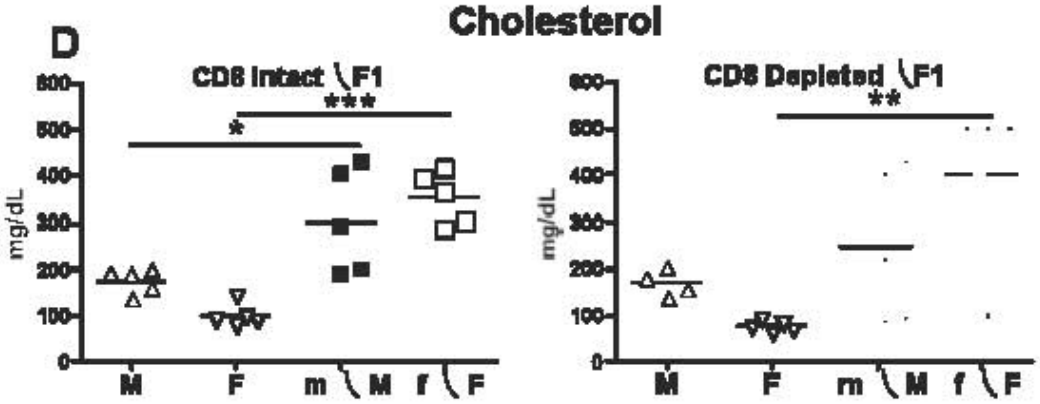
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Fig. 2

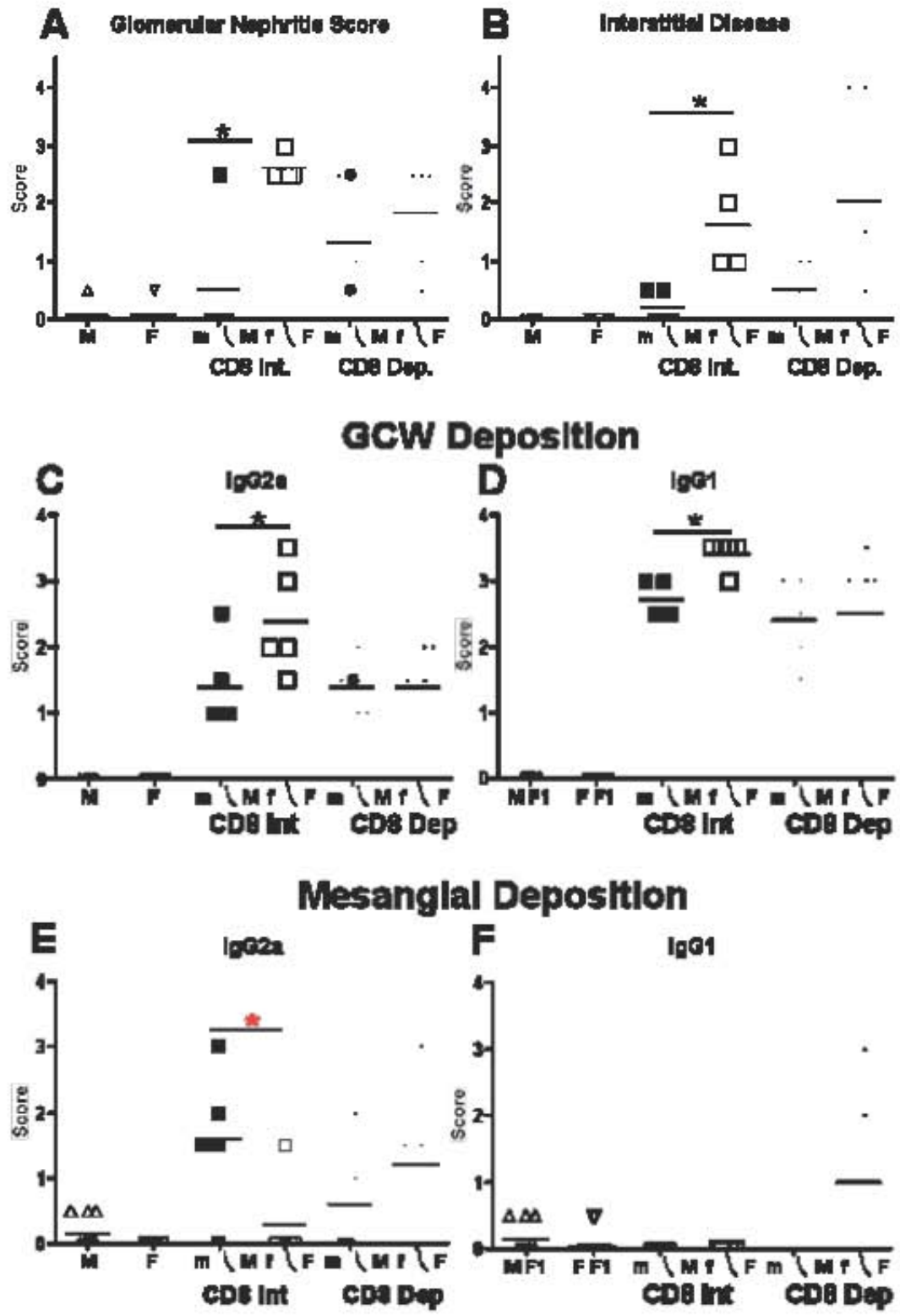
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Fig. 3

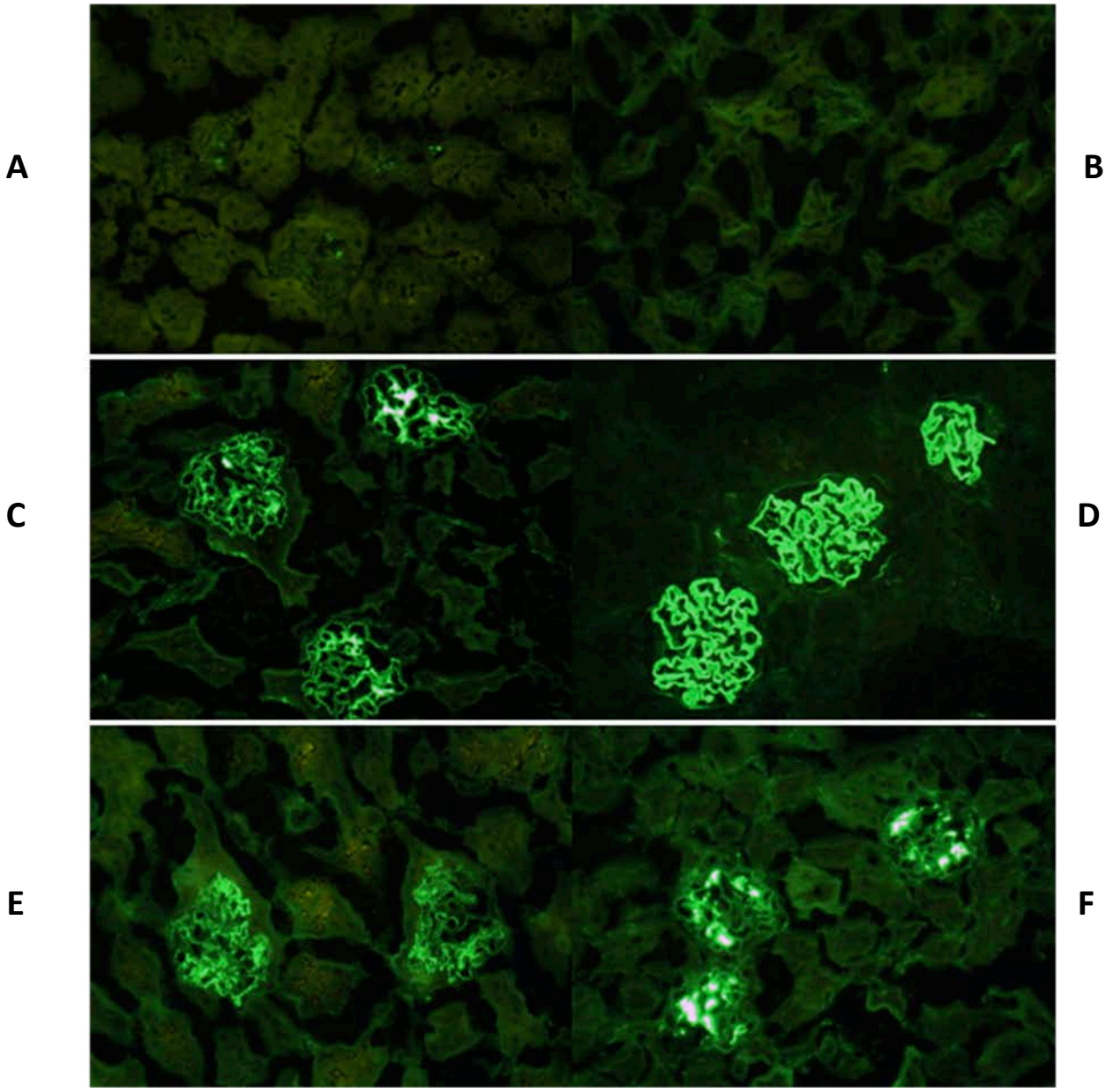
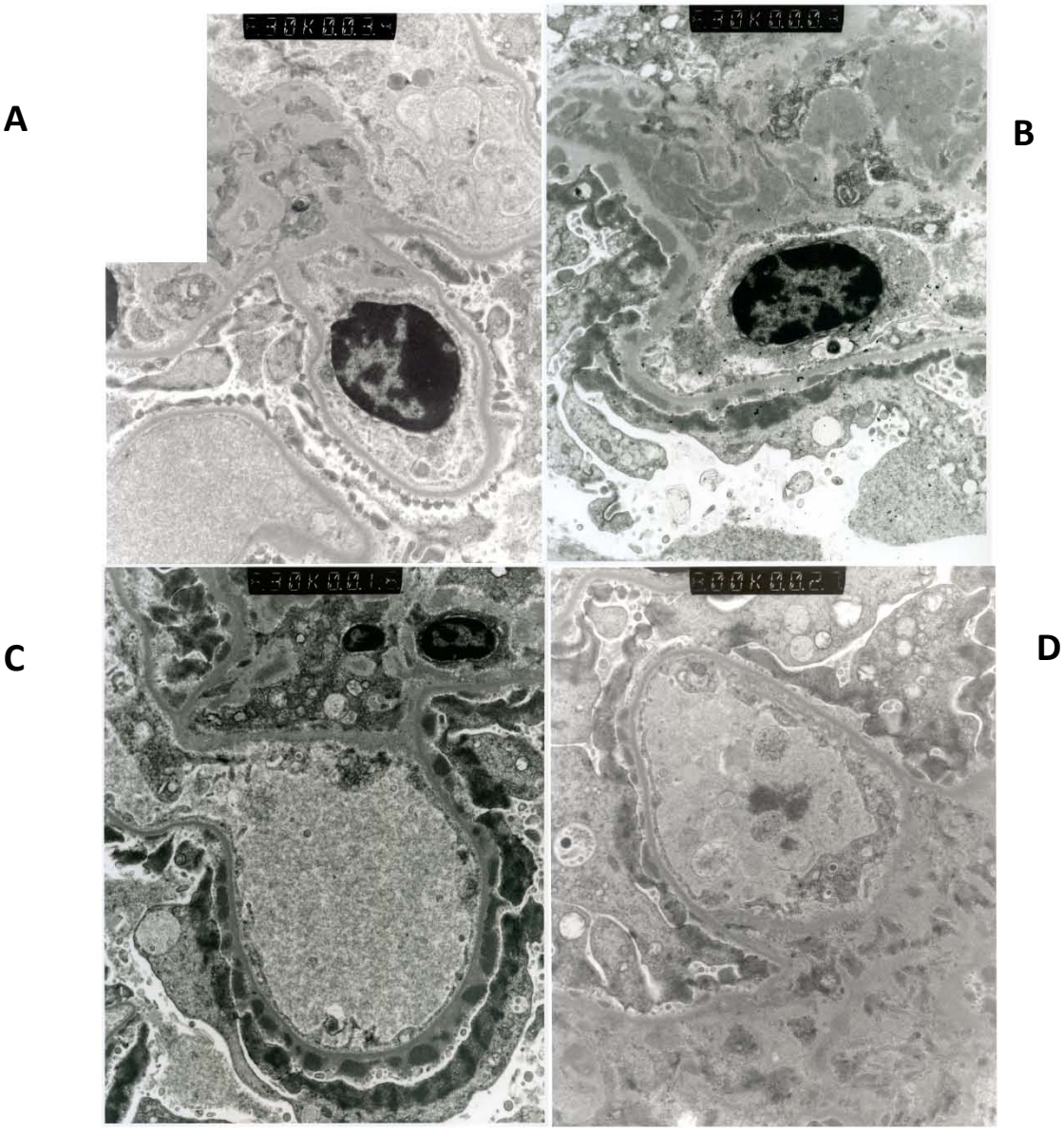


Fig. 4



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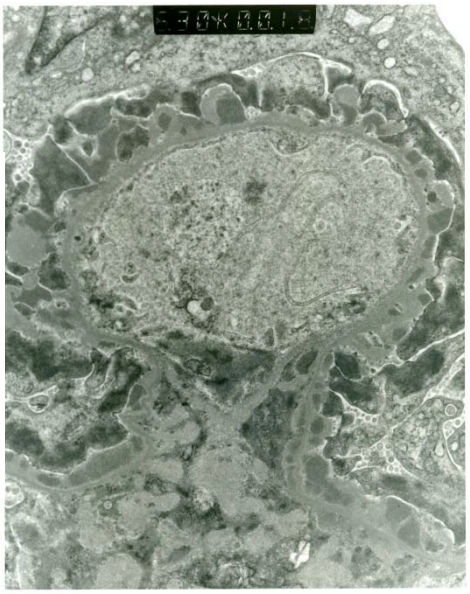


Fig. 5

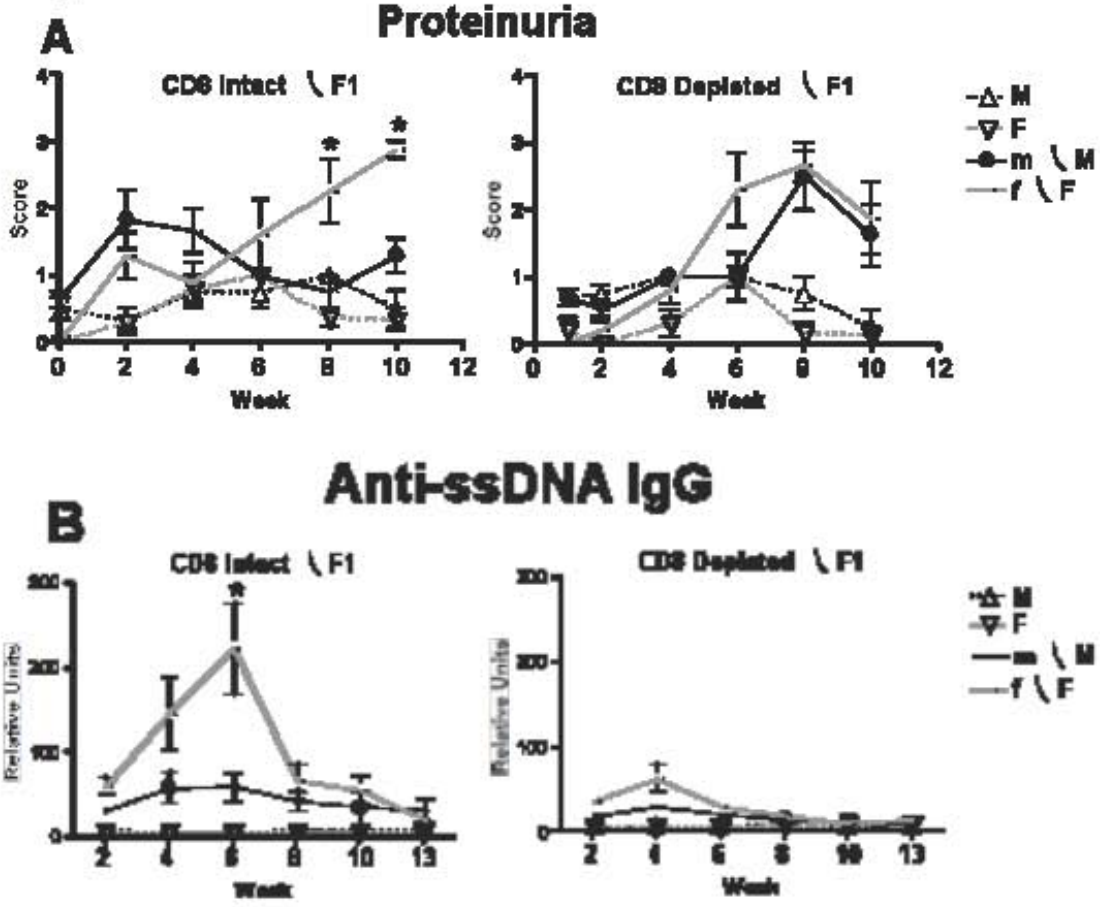

A
Proteinuria
CD8 Intact \ F1
CD8 Depleted \ F1
Score
Week
M
F
m \ M
f \ F
B
Anti-ssDNA IgG
CD8 Intact \ F1
CD8 Depleted \ F1
Relative Units
Week
M
F
m \ M
f \ F

Fig. 6

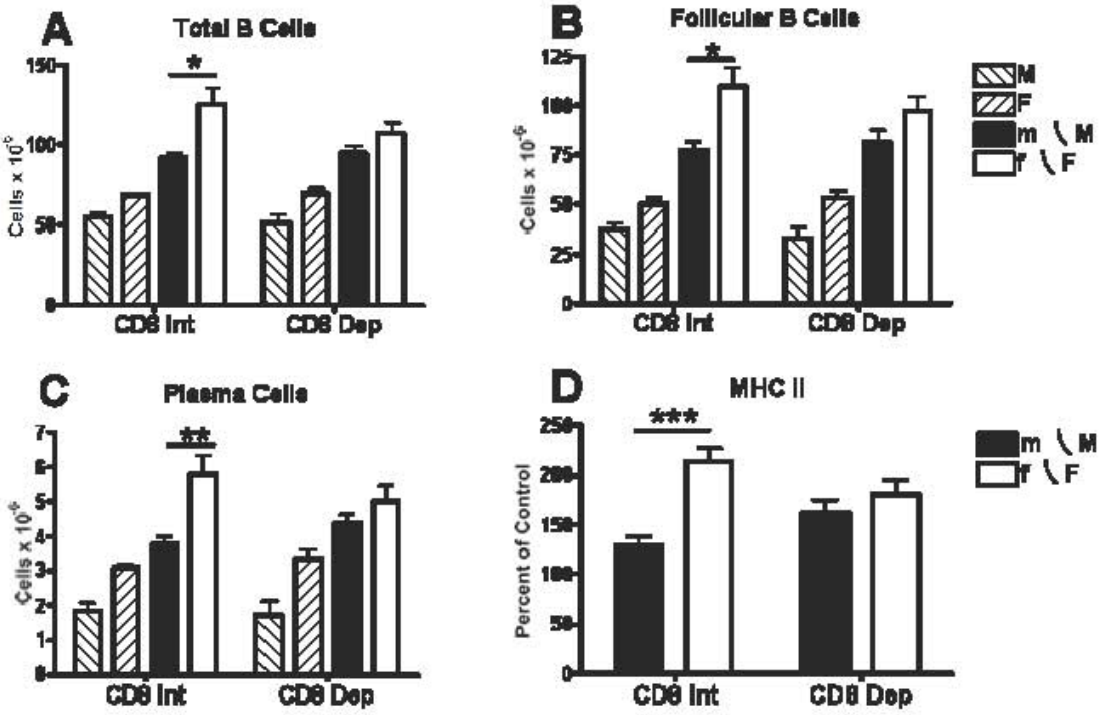
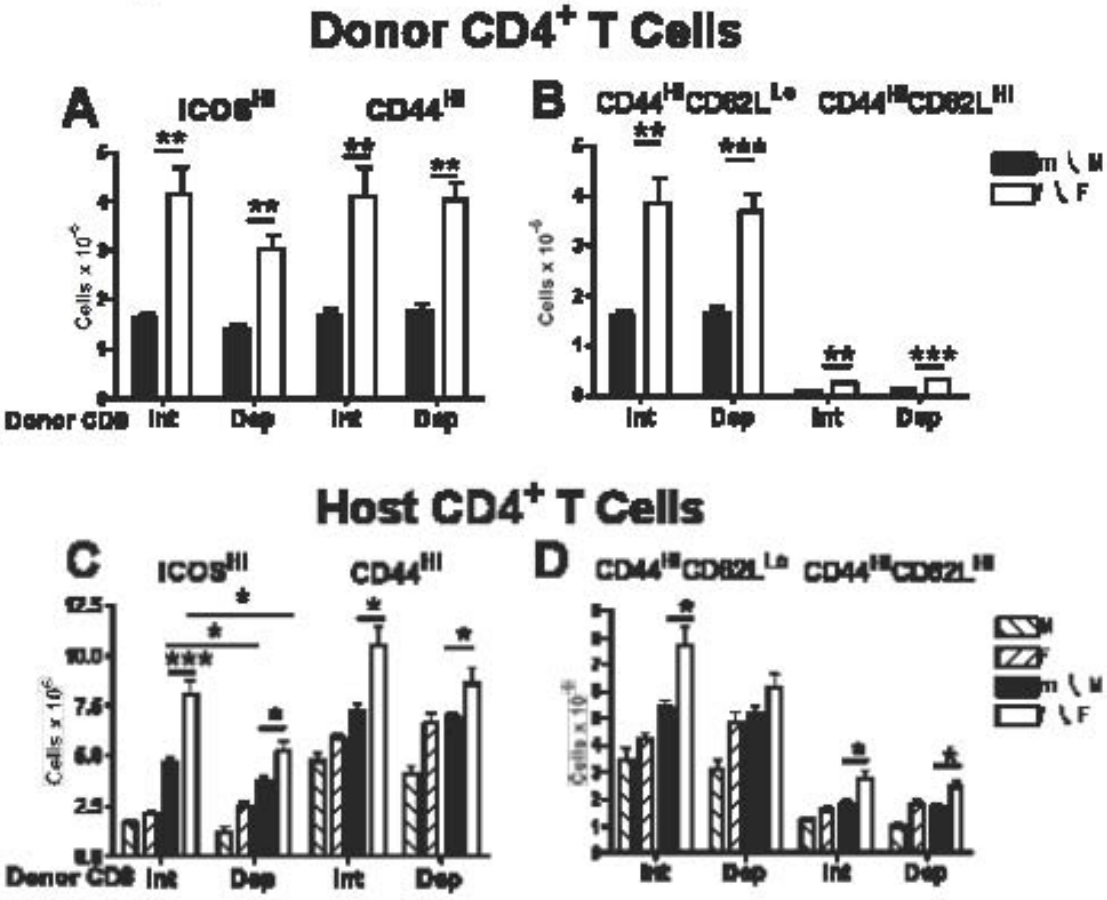

A
Total B Cells
*
Cells x 10^-6
CD8 Int
CD8 Dep
B
Follicular B Cells
*
M
F
m \ M
f \ F
C
Plasma Cells
**
D
MHC II
***
Percent of Control

Fig. 7


Donor CD4+ T Cells
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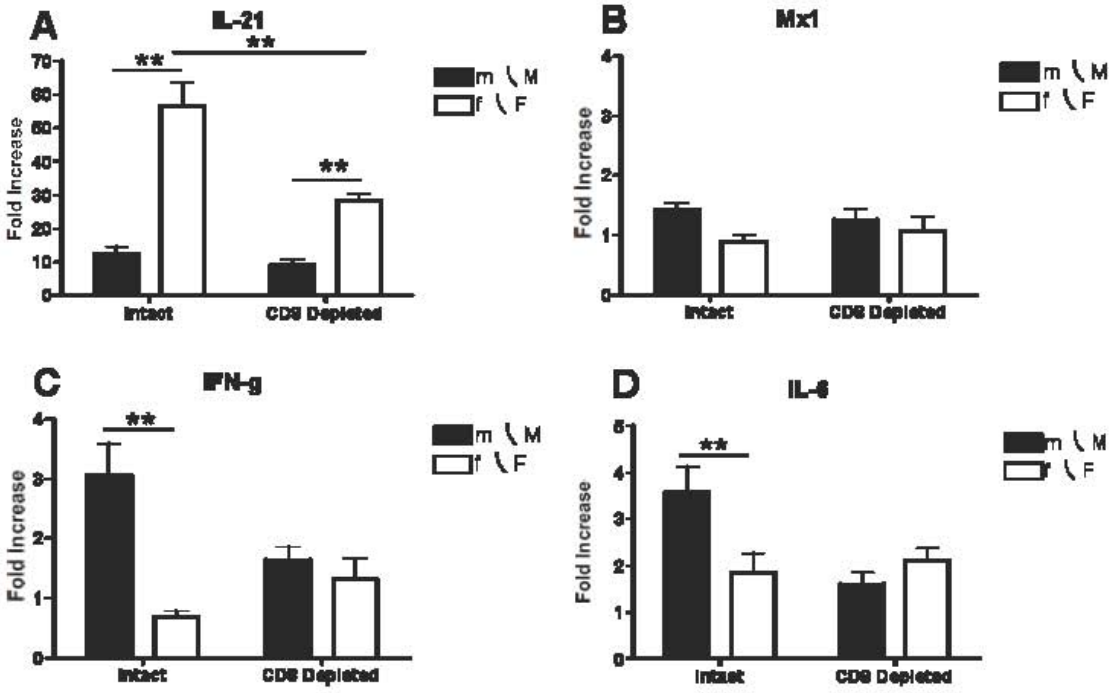
ICOS^HI
CD44^HI
B
CD44^HICD62L^Lo
CD44^HICD62L^HI
Cells x 10^-6
Donor CD8
Int
Dep
Int
Dep
m \ M
f \ F
Host CD4+ T Cells
C
ICOS^HI
CD44^HI
D
CD44^HICD62L^Lo
CD44^HICD62L^HI
Cells x 10^-6
Donor CD8
Int
Dep
Int
Dep
M
F
m \ M
f \ F

Fig. 8


A
IL-21
**
**
**
Fold Increase
70
60
50
40
30
20
10
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Intact
CD8 Depleted
m \ M
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Fold Increase
4
3
2
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IFN-g
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Fold Increase
4
3
2
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Intact
CD8 Depleted
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f \ F
D
IL-6
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Fold Increase
5
4
3
2
1
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Intact
CD8 Depleted
m \ M
f \ F

Chapter 3

Donor CD8 T cell activation is critical for sex based differences in lupus-like disease in chronic GVHD mice: II. Persistence of donor CD8 T cells in females is associated with prolonged donor CD4 T cell proliferation and greater engraftment[1].

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Keywords: graft-vs-host disease, Fas, costimulation, lupus

Abstract

We used the DBA→F1 model of induced lupus to investigate sex based differences in disease severity. In this model, disease severity parallels the strength of the donor CD4 T cell response to host MHC II and greater engraftment of donor CD4 T cells at two weeks in females is associated with greater severity of lupus like renal disease long term. Donor CD8 T cells are not thought to play a role. Based on previous work demonstrating no sex based differences in donor T cell activation and expansion from days 0 to 7 after transfer, we performed a kinetic analysis of donor and host T cells during the second week after transfer. Surprisingly, we observed that depletion of donor CD8 T cells prior to transfer significantly reduces sex based differences in donor CD4 T cell engraftment at two weeks. Kinetic analysis from days 8-12 indicated that m→M mice have a stronger donor anti host and a host anti-donor response as shown by earlier host B cell elimination, greater donor CD8 numbers at day 8 and 10 at , NK increase and at day 8 and greater host CD8 proliferation in males. The presence of donor CD8 T cells increases the HVG for both sexes but is greater for males which in turn is associated with greater and more complete donor CD8 downregulation. The longer persistence of donor CD8 T cells in females is associated with continued proliferation and greater engraftment at two weeks. Greater female engraftment of donor CD4 T cells is CD8 dependent and linked to greater day 8 GVH and HVG in males as depletion of donor CD8 T cells eliminates both of these sex based differences. These results indicate a role for sex based differences in CD8 T cell activation in shaping lupus like disease expression.

Note: Technical assistance on some experiments was provided by R. Puliaev, I. Puliaeva, or K. Soloviova.

Introduction

Human systemic lupus erythematosus exhibits a striking female predominance, particularly during the child bearing years, in the range of 8-10 fold greater incidence compared to males (1). This long standing observation has been an important but poorly understood clue regarding disease pathogenesis implicating sex hormones in disease expression (2). Murine models of lupus have been of enormous benefit in unraveling the disordered immunoregulation characteristic of lupus, however many models do not exhibit female skewing of disease. An important exception is the NZB/W model of spontaneous murine lupus in which landmark hormonal depletion and add-back studies demonstrated that female sex hormones accelerate disease and androgens retard disease expression (reviewd in (3). Despite major advances in our understanding of hormonal influences on the functioning of a normal immune system (4), the exact role played by female sex hormones in lupus pathogenesis is not clear.

In contrast to the spontaneous development of lupus in NZB/W mice, the p→F1 model is an induced model of lupus in which the transfer of parental CD4 T cells into a normal semi-allogeneic F1 results in B cell hyperactivity, autoantibody production and lupus like renal disease. Sex based differences are best documented in the DBA→B6D2F1 following the transfer of unfractionated splenic DBA donor cells (5). A severe renal disease to include features of nephrotic syndrome was first shown using multiple transfers of unfractionated splenic and lymph node lymphocytes however the severity in males in relation to females was not examined in those studies (6, 7).

Previous work has also shown a critical role for donor CD4 but not CD8 T cells in mediating lupus like disease in the p→F1 model (8-10). Moreover, disease severity and autoantibody levels are directly proportional to the number of transferred donor CD4 T cells (7,

11). In the DBA→F1 model of lupus like renal disease it has been shown that two weeks after a single transfer of 80 x 10^6 unfractionated DBA splenocytes (containing approximately 10 - 12 x 10^6 CD4 T cells) into BDF1 mice, engraftment of donor CD4 T cells is 2-3 fold greater in f→F than in m→M GVHD mice whereas engraftment of donor CD8 T cells is minimal in both groups (5). This increase female donor CD4 T cells at two weeks is a surrogate marker for greater lupus like renal disease in females long term. No sex based differences in donor T cell homing or initial activation were observed prior to day 7 and both male and female donor CD4 T cells exhibit increased proliferation at day 7 after transfer. Importantly, sex based differences in donor T cell engraftment at two weeks segregate with the sex of the host and not the donor (5). Thus, differences in donor and host T cell activation kinetics after day 7 appear to be central to sex based differences in lupus like disease severity long term. Accordingly, in these studies we characterized donor and host lymphocyte kinetics from days 8-12 to determine the mechanism involved in greater female donor CD4 T cell engraftment at day 14. We demonstrate that sex based differences in donor CD4 T cell engraftment are critically dependent on co-injection of donor CD8 T cells that promote greater donor CD4 T cell proliferation and expansion than seen in the absence of donor CD8 T cells. Additionally, donor CD8 T cells induce an earlier and stronger donor anti-host and host anti-donor T cell response in males that results in more complete donor CD8 T cell contraction and conversely greater survival of donor CD8 T cells in females which in turn promotes donor CD4 T cell expansion. Thus, the relatively longer survival of donor CD8 T cells in females is associated with greater donor CD4 T cell engraftment.

Materials and Methods

Mice: 6-8 week old male and female DBA/2J (DBA) (H-2^d) and B6D2F1 (BDF1)(H-$2^{b/d}$) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). All animal procedures were pre-approved by the Institutional Animal Care and Use Committee at the Uniformed Services University of Health Sciences.

Induction of GVHD: Single cell suspensions of DBA splenocytes were prepared as described (12) and transferred into BDF1 hosts by tail vein injection. Donor and hosts were age and sex matched such that male donors were transferred into male hosts (m→M) and female donors into female hosts (f→F). Donor populations were analyzed by flow cytometry to determine the percentage of CD4, CD8 T cells in the donor inoculum prior to transfer. The number of donor CD4 and CD8 T cells injected are indicated in the text and respective figure legends. For CD8 depletion studies, CD8 T cells were positively selected and removed from the donor population using magnetic beads purchased from Invitrogen (Carlsbad, CA). The resulting cells contained <1% contaminating DBA CD8 T cells.

Flow cytometric analysis. Spleen cells were first incubated with anti-murine Fcγ receptor II/III mAb, 2.4G2 for 10 min and then stained with saturating concentrations of Alexa Fluor 488-conjugated, biotin-conjugated, PE-conjugated, APC-conjugated, PerCPCy5.5-conjugated, Pacific Blue-conjugated, and Pacific Orange-conjugated mAb against CD4, CD8, B220, H-$2K^b$, I-A^b, CD44, CD62L, ICOS (CD278), CD80, CD86, CD21, CD23, and CD138 were purchased from either BD Biosciences (San Jose, CA), BioLegend (San Diego, CA), eBiosicnce (San Diego, CA), or Invitrogen (Carlsbad, CA). Biotinylated primary mAb were detected using either

streptavidin-PE-Texas Red (BD Bioscience) or streptavidin-Alexa Fluor 700 (Invitrogen). Cells were fixed in 1% paraformaldehyde. Multi-color flow cytometric analyses were performed using a BD FACScan flow cytometer or BD LCRII flow cytometer (BD Biosciences, San Jose, CA). Lymphocytes were gated by forward and side scatter and fluorescence data were collected for a minimum of 10,000 gated cells. Studies of donor T cells were performed on a minimum of 3,000 gated cells that were positive for $CD4^+$ or $CD8^+$ and negative for MHC class I of the uninjected parent.

KI-67 methodology

A PE-anti-human mAb against KI-67 was purchased from BD Biosciences (San Jose, CA). Intracellular staining for KI-67 was performed using the Foxp3 buffer staining set from eBioscience (San Diego, CA) according to kit protocol.

Cytokine Expression by PCR: RNase-free plastic and water were used throughout the assay. Splenocytes (1×10^7) were homogenized in 1 ml of RNA-STAT-60 (Tel-Test, Friendswood, TX). RNA samples were reverse transcribed with Moloney murine leukemia virus reverse transcriptase (Invitrogen, Carlsbad, CA). Primers and probes were purchased from Applied Biosystems International (ABI). All real time PCR primers were purchased from Applied Biosystems.

Statistical Analysis. Statistical comparisons (t tests and linear regressions) were performed using Prism 4.0 (Graphpad Software)**.** Experimental groups were compared using two tailed t test unless otherwise noted.

Results

Greater day 14 engraftment of donor CD4 T cells in female DBA→F1 mice is correlated with greater day 14 engraftment of donor CD8 T cells. To determine the mechanism by which f→F mice exhibit greater donor CD4 T cell engraftment at two weeks after transfer and, as a consequence, more severe renal disease compared to m→M mice (5), we examined donor T cell engraftment kinetics. Because sex based differences in donor T cell homing, activation or engraftment are not present during the first week after transfer (5), we examined donor and host lymphocyte kinetics during the second week after transfer using the previously published single dose protocol shown to result in sex based differences in engraftment and renal disease i.e. a single dose of ~ 80 – 90 x 10^6 unfractionated DBA splenocytes typically containing ~10-14 x 10^6 DBA CD4 T cells. Severity of lupus-like disease in the DBA→BDF1 model is directly related to the number of donor CD4 T cells injected (7, 11) prompting us to eliminate potential variability by standardizing donor inocula based on the number of CD4 T cells rather than by total splenocyte number. We observed in preliminary experiments that a donor cell inoculum containing 10 x 10^6 DBA CD4 T cells was $\leq$ the threshold for GVHD induction resulting in high mouse-to-mouse variability in engraftment to include occasional engraftment failures. We therefore examined the post-injection kinetics for two higher but very close doses of DBA CD4 T cells that still remained within the previously published range of donor splenocytes resulting in greater female disease severity: a) 12 x 10^6 CD4 donor cells (typically 80-90 x 10^6 splenocytes) and containing 3.9 x 10^6 DBA CD8 T cells (Fig. 1A,C) or b) 14 x 10^6 DBA CD4 donor cells (typically 90-100 x 10^6 splenocytes and containing 4.3 x 10^6 DBA CD8 T cells) (Fig 1B,D). Days 8, 10, 12 and 14 were

examined for the 14 x 10^6 dose. These same time points were examined for the 12 x 10^6 dose with the exception of day 14 engraftment which has been previously published at this dose (5). Both doses result in greater lupus-like renal disease severity for f→F mice (5) (ms. in preparation).

The engraftment kinetics of donor CD4 T cells during the second week after transfer are shown in Figs. 1A, B. For both donor cell doses, donor CD4 T cell engraftment is slightly but significantly greater in m→M mice vs. f→F mice at days 8 and 10 (Figs.1A, B) however between days 10 and 12, these curves cross as male numbers decline and female numbers increase. By day 14, f→F mice exhibit 2.5 fold greater ($p < 0.01$) donor CD4 T cell engraftment at the 14 x 10^6 dose (Fig. 1B), a proportion similar to the ~3-fold increase previously reported at day 14 for the 12 x 10^6 dose (5).

The engraftment kinetics of donor CD8 T cells (Fig. 1C,D) parallel those of donor CD4 T cells in that there is significantly greater engraftment in m→M mice at days 8 and 10. The difference is greater at the 14 x 10^6 dose (~2.5 fold) (Fig. 1D) vs. the 12 x 10^6 dose (<2-fold) (Fig. 1C). Nevertheless, as with donor CD4 T cell engraftment, the CD8 engraftment curves for m→M and f→F cross from days 10 –12 as male CD8 T cells undergo homeostatic contraction whereas female donor CD8 T cells exhibit expansion, albeit highly variable and thus not statistically significant.

Because of the high mouse to mouse variability in donor CD8 T cell engraftment in f→F mice, linear regression analysis were performed comparing engraftment of donor CD4 and CD8 T cells for m→M and f→F mice for the results using the 14 x 10^6 dose (Figs 1B,D). Both male and female groups exhibited weak correlation ($r^2 < 0.4$) at day 8

(Fig. 2 A, E) but a strong correlation ($r^2 > 0.8$) at day 10 (Fig. 2B, F). After day 10, sex based differences in engraftment are seen and a strong correlation between engrafted CD4 T cells and CD8 T cells persists only in f→F mice ($R^2 > 0.8$) (Figs 2 C-D, G-H). Based on this correlation, unless otherwise noted, the following studies examine the mechanism of sex based differences using the 14 x 10^6 dose of donor CD4 T cells.

Sex based differences in donor CD4 T cell engraftment in DBA→F1 mice are critically dependent on co-injection of donor CD8 T cells. The foregoing data raise the possibility that sex based differences in donor CD4 T cell engraftment at two weeks after transfer have a CD8 component i.e. that co-injection of donor CD8 T cells influences donor CD4 T cell engraftment at day 14, particularly for f→F mice. To directly address this question, we compared the kinetics of donor CD4 engraftment following the transfer of DBA splenocytes depleted only of CD8 T cells (CD8 depleted→F1) for both m→M and f→F mice at a dose of 14 x 10^6 CD4 T cells. As shown in Fig.3, CD4 engraftment kinetic curves for m→M and f→>F mice closely resemble each other at all time points. Although female CD4 engraftment was significantly greater at days 10 and 14, the striking 2-3 fold greater day 14 female CD4 engraftment previously published (5) and shown in Fig 1B (2.5 fold) is reduced to 1.2 fold if CD8 T cells are depleted from the donor inoculum. This near equalization is due in part to changes in both male and female CD4 engraftment. Specifically, for males at day 14, CD8 depleted m→M mice exhibit engraftment of donor CD4 T cells that is slightly but not significantly increased compared to CD8 intact (CD8 intact→F1) donor splenocytes (Fig. 3). In contrast, the

elevated day 14 engraftment of donor CD4 T cells (~ 10 x 10^6) seen for CD8 intact f→F mice (Fig.1B) is significantly reduced (1.6 fold, $p<0.05$ 1 Tail) for CD8 depleted→F1 mice although the latter values remain significantly greater than that of CD8 depleted m→M mice.

Co-injection of donor CD8 T cells is associated with greater proliferation of donor CD4 T cells: To further address a potential role for donor CD8 T cells in promoting sex based differences in donor CD4 T cell engraftment at two weeks, we examined donor T cell proliferation by flow cytometric assessment of KI-67 expression, a cell cycle protein only found in cells that are actively undergoing cellular proliferation (G1, S, G2, and mitosis) and is not detectable in resting (G0) cells (13)**.** Using a donor cell dose of 14 x 10^6 CD4 T cells, the kinetics of donor T cell proliferation were compared for both CD8 intact→F1 and CD8 depleted →F1 mice. The engraftment data for these cohorts is shown in Figs 1B, D and Fig. 3. Co-injection of donor CD8 T cells boosts the proliferative response of donor CD4 T cells at days 8 and 10. This effect is best seen for m→M mice where a significant increase in both the percentage and number of proliferating donor CD4 T cells is seen at days 8 and 10 for CD8 intact→F1 vs. CD8 depleted→F1 mice. A similar boosting effect is seen for f→F mice but is less pronounced. Specifically, CD8 intact f→F mice exhibit significantly greater percentage but not number of proliferating donor T cells at days 8, 10, and 12 vs. CD8 depleted→F1 mice. There are no sex based differences in donor CD4 T cell proliferation at any time point tested for CD8 depleted →F1 mice whereas for CD8 intact→F1 mice, males exhibit significantly greater proliferation (both percentage and number) at day 8 however by day 10 these sex based differences are lost. As with the respective engraftment curves (Figs 1B, D), the proliferation curves cross for CD8 intact →F1 mice from days 10 to 12. By day 12, the percentage and numbers of proliferating CD4 T cells in CD8 intact m→M mice have declined to levels comparable to levels of both male and

female CD8 depleted→F1 mice. By contrast, the percentage (but not number) of proliferating donor CD4 T cells in CD8 intact f→F mice was significantly greater than that of CD8 depleted f→F mice. Thus, the major effect of donor CD8 T cells is seen for male mice in which there is significantly greater donor CD4 T cell proliferation (both numbers and percentage) at days 8 and 10 compared to male CD8 depleted →F1 mice. The significantly greater donor CD4 proliferation at day 8 for CD8 intact m→M mice vs. CD8 intact f→F mice is in agreement with greater day 8 donor CD4 engraftment in m→M mice (Fig. 1B) and consistent with a greater donor anti-host response in males.

Males also exhibit greater proliferation of donor CD8 T cells at day 8 compared to females as shown by both the percentage (Fig. 4C) and numbers (Fig. 4D) of proliferating cells. These values are not significantly different at day 10 and from days 10-12, these curves cross yielding a non-significant trend toward greater female percentage and number at day 12.

Together these data demonstrate that the presence of co-injected donor CD8 T cells boosts donor CD4 proliferation and engraftment best for males from days 8-10 and days 10-12 for females. The greater proliferation of male donor CD8 T cells at day 8 is consistent with a stronger donor anti-host CTL response in m→M mice vs f→F mice. These results support the conclusion that sex based differences in donor CD8 activation and engraftment are associated with sex based differences in donor CD4 T cell engraftment.

Host T and B cell kinetics: Differences in the relative strength and timing of donor T cell activation can be assessed either directly as in Figs 1-4 or indirectly by examining the changes in host T and B cells during the first two weeks after donor cell transfer. Following donor cell transfer, there is an initial expansion of host cells driven largely by

donor CD4 T cell activation of host B cells seen as early as day 3 and sustained to day 14 if donor CD8 T cells are not co-injected (14). The co-injection of donor CD8 T cells with CD4 T cells results in a curtailment of B cell expansion at ~ day 7 as donor CTL effectors mature and eliminate host cells, particularly B cells. Following maturation, contraction of donor CD8 CTL typically occurs between days 12-14 allowing the potential for re-expansion of host cells, particularly B cells, that escaped the initial wave of CTL mediated eradication (12, 14). Thus, if CD8 intact m→M mice exhibit an earlier donor anti-host response as suggested by Figs 1B, D and Fig 4, then m→M mice should also exhibit greater elimination of host cells, particularly B cells at day 8 compared to CD8 intact f→F mice. The kinetics of host B and T cell numbers from days 8-14 are shown in Fig. 5 for the CD8 intact→F1 and CD8 depleted→F1 cohorts receiving 14 million cells (Figs 1B, D and Fig 3 respectively). As shown in Fig. 5A, CD8 depleted →F1 mice exhibit significant expansion of B cells over control uninjected F1 values at all time points from days 8-14 and are in the range of ~1.5-3 fold over control. There is no evidence of an elimination phase in CD8 depleted →F1 mice as B cell expansion seen at day 8 remains relatively constant out to day 14. These results are consistent with donor CD8 depletion and the absence of effector CD8 CTL.

By contrast, CD8 intact→F1 mice exhibit differences compared to CD8 depleted→F1 and sex based differences within the CD8 intact→F1 group. For example, at days 8 and 10 CD8 intact m→M mice exhibit B cell numbers that are not significantly different from control uninjected F1 mice and importantly are significantly reduced compared to both groups of CD8 depleted→F1 mice. These reduced B cell values for CD8 intact m→M mice are consistent with B cell elimination by donor anti-host CD8

CTL**.** Sex based differences for CD8 intact→F1 mice are seen at day 8 in that CD8 intact f→F mice exhibit significant elevation of host B cells compared CD8 intact m→M mice However by day 10, host B cells numbers in CD8 intact f→F mice decline to levels that do not differ significantly from those of CD8 intact m→M mice and were significantly lower than that of uninjected F1 mice. The reduction in host B cells at day 10 seen for both groups of CD8 intact→F1 mice vs CD8 depleted→F1 mice is consistent with donor CD8 CTL anti-host CTL mediated elimination, albeit incomplete, of host B cells as previously described. The delay in host B cell elimination for f→F mice vs m→M mice is further evidence supporting an earlier donor anti-host CD8 CTL response in males. As shown in Fig. 1D, donor CD8 T cells in both CD8 intact →F1 groups undergo contraction from days 10-14 which is associated with a rebound increase in host B cells for both male and female CD8 intact→F1 mice. As a result, by day 14 host B cells have completely rebounded and are not significantly different from CD8 depleted→F1 donor cells.

The kinetics of host CD4 T cells shown in Fig. 5B mirror the results of host B cells. Notably, at day 8 CD8 intact m→M mice exhibit host CD4 T cell numbers that are significantly reduced compared to either CD8 intact f→F mice, CD8 depleted m→M, or even uninjected F1 mice**.** By day 10, values for CD8 intact f→F mice have declined to those for CD8 intact m→M mice consistent with anti-host CTL activity in vivo that is delayed compared to CD8 intact m→M mice. From days 10-14, host CD4 T cells rebound to levels comparable for CD8 depleted→F1 mice consistent with homeostatic downregulation of donor CD8 CTL effectors. Host CD8 T cells are much less susceptible to elimination by donor CTL than are host B cells and CD4 T cells (12) and as shown in Fig. 5C, do not exhibit significant changes between groups from days 8-14

however all groups exhibit expansion between days 10 and 14 typical of chronic GVHD in this model (12).

These results in CD8 intact→F1 mice are consistent with previous work demonstrating that in DBA→F1 mice the strength of the donor anti-host CTL response is substantially less than that seen for B6→F1 mice (15) and that although homeostatic downregulation of CD8 CTL effector activity occurs in both DBA→F1 and B6→F1 mice at roughly the same time (days 10-14), B cell elimination is incomplete only in DBA→F1 mice allowing their re-expansion and evolution to lupus like disease (14**).** The data in Fig 5 demonstrate sex based differences at day 8 in host B and T cell numbers for CD8 intact→F1 mice are consistent with an earlier, and perhaps stronger donor anti-host CTL response in males compared to females.

Sex based differences in F1 anti-parent responses parallel sex based differences in the parent-anti F1 response. A counter-regulatory F1 anti-parent or host-vs-graft (HVG) response is well described in the p→F1 model not only for acute GVHD (14) but also for the DBA→F1 model of chronic GVHD in which elimination of donor cells by host CD8 CTL and NK cells has been demonstrated (14). The relatively weaker F1 anti-parent response mitigates but does not prevent the stronger parent anti-F1 response (14**).** If the parental anti-F1 response in CD8 intact m→M mice is earlier than that of f→F mice this should be accompanied by an earlier stronger F1 anti-parent response. Host CD8 T cell proliferation is shown in Fig. 6 at days 8, 10 and 12 for CD8 intact→F1 and CD8 depleted→F1 mice. At day 8, CD8 intact m→M mice exhibit significantly greater host CD8 T cell proliferation, either by percentage or numbers (Figs. 6A, B) compared to either CD8 intact f→F mice, CD8 depleted →F1 mice or control F1 mice. By day 10, sex based differences are lost for CD8 intact→F1 mice as CD8 proliferation (both numbers

and percentages) increases in f→F mice to levels equivalent for CD8 intact m→M mice. Representative flow cytometry tracings are shown in Figs. 6C-F

These results mirror the delay in donor anti-host CTL elimination of host cells seen for f→F vs m→M mice (Fig 5) and support the conclusion that m→M mice exhibit an earlier parent anti-F1 response. Importantly, at day 10 both CD8 intact m→M and f→F mice exhibit significantly greater host CD8 T cell proliferation (both percentage and numbers) than CD8 depleted →F1 mice or uninjected control mice indicating that host CD8 T cell proliferation is strikingly and significantly dependent on the injection of donor CD8 T cells. Nevertheless, a low level but significant increase in both percentage and numbers of proliferating host CD8 T cells is also seen for CD8 depleted→F1 mice compared to uninjected control F1 mice. Thus, the host CD8 T cell HVG response consists of a low level donor CD4-dependent response and a significantly greater donor CD8 dependent response.

By day 12, the percentage of proliferating host CD8 T cells has peaked for CD8 intact→F1 mice and all groups, both intact→F1 and depleted→F1 mice exhibit an increase in proliferating host CD8 T cell numbers simultaneously with contraction of donor CD8 T cells (Fig. X) and the transition in phenotype to chronic GVHD (12, 14). These results demonstrate that not only is the CD8 T cell HVG response strikingly dependent on the presence of donor CD8 T cells but also that sex based differences in the F1 anti-parent response are seen for CD8 intact transfers that mirror the parent anti-F1 response, i.e. m→M mice exhibit an earlier donor anti-host and host anti-donor CD8 T cell response as measured by greater proliferation and numbers Confirmatory evidence of sex based differences in the F1 anti-parent response are seen in the numbers of host NK cells. CD8 intact m→M exhibit a significant increase in host NK cells (Fig. 7) at day 8 compared to either CD8 intact f→F ($p<0.001$) or uninjected control F1 mice ($p<0.1$).

CD107a is a marker of recent degranulation and CTL activity (16) and its expression on host CD8 T cells at day 10 is greater in m→M vs. f→F mice consistent with a stronger F1 anti-parent response in males. The greater percentage of donor CD8 T cells that are CD107ahi in males is also consistent with the foregoing evidence of a stronger but waning parent anti-F1 response in males at day 10. These results further support the conclusion that for CD8 intact→F1 mice, m→M mice exhibit a stronger GVH response that is associated with a stronger HVG response compared to CD8 intact f→F mice.

CD8 Intact m->M exhibit increased cytokine expression vs. f->F at day 8: Cytokines important in CD8 CTL activation were examined for sex based differences during days 8-14. In panels A-F, the kinetics of IFN-a related genes Mx-1, OAS and IP-10 (both IFN-a and IFN-g) are shown for CD8 intact→F1 (Fig 8A, C, E) and CD8 depleted →F1 (Fig. 8B,D, F) mice. Three observations can be made from Fig. 8. Firstly, for both sexes, CD8 intact →F1 mice exhibit a peak at day 10 for all three genes followed by rapid downregulation at day 12. Secondly, the day 10 peak is significantly greater in males vs. females for Mx-1 and IP-10 Lastly, CD8 depleted→F1 mice exhibit flattened curves with either a day 10 peak that is either non-existent or much reduced. In particular, the reduction in day 10 peak Mx-1 expression in CD8 depleted→F1 mice is significantly greater for m→M mice than for f→F mice. Although these cytokine kinetics performed on whole spleens do not distinguish host from donor contributions, they nevertheless further support the conclusion that the GVH and HVG reactions from days 8 to 10 are stronger in males than females for CD8 intact→F1 mice and are markedly attenuated in CD8 depleted→F1 mice.

Although IL-2 is expressed at very low levels in both groups, it nevertheless parallels the kinetics seen in Figs 9 A-F. 1. Although IFN-g gene expression exhibits a different kinetic curve

from the IFN-a genes, there is nevertheless greater day 10 IFN-g expression in males vs. females for CD8 intact→F1 mice and little expression in CD8 depleted→F1 mice.

IL-21 has both a CD8 CTL promoting and B cell promoting properties (17). The kinetics of IL-21 gene expression mirrors those of IFN-a inducible genes from days 8-10 but afterwards they differ. Specifically, from days 8-12, CD8 intact→F1 exhibit similar IL-21 expression with a peak at day 10 followed by a decline at day 12 that is significantly lower for m→M mice. . A second peak at day 14 is observed and at both days 12 and 14, f→F mice exhibit significantly greater IL-21 gene expression compared to m→M mice. By contrast, CD8 depleted f→F mice exhibit a reversal of the sex based differences seen for the previous cytokines in that the day 10 peak is significantly greater for f→F vs m→M mice. Values then decline in both groups and no further sex based differences are observed. Importantly, by day 14 sex based differences in IL-21 gene expression are seen only in CD8 intact →F1 mice (i.e. increased in f→F mice) corresponding to the significantly greater day 14 donor CD4 engraftment in CD8 intact f→F vs m→M mice (Fig. 1B). Taken together, these results support the conclusion that the cytotoxic HVG and GVH response from days 8-10 is stronger in males and is lost if CD8 T cells are depleted from the donor inoculum. As the GVH and HVG terminate by day 12, an increase in IL-21 expression from days 12-14 is seen particularly in CD8 intact f→F mice.

Discussion

A central role for CD4 T cells in the pathogenesis of both human and murine lupus is well documented (18). Similarly, in the p→F1 model, donor CD4 T cells are both necessary and sufficient for lupus like disease induction as a result of recognition of host (foreign) MHC II (9, 10). Severity of lupus like disease in the DBA→F1 transfer is directly related to the number of DBA splenocytes, particularly CD4 T cells transferred (7)**.** Moreover**,** greater long term severity of renal disease in females is associated with greater female engraftment of donor CD4 T cells at two weeks that persists long term. Because of the central role of donor CD4 T cells in disease expression, the greater female engraftment at two weeks is an important surrogate marker and predictor of long term lupus severity. An essential question then is what accounts for the early sex based differences in engraftment of donor CD4 T cells despite the transfer of equivalent numbers? It has been previously demonstrated that DBA→F1 mice exhibit no sex based differences in donor T cell homing, initial activation or engraftment from days 0-7 after donor transfer. This study therefore focused on the second week after donor transfer and makes two novel observations: 1) greater female engraftment of donor CD4 T cells at two weeks is preceded at day 8 by a stronger GVH and reciprocal HVG in males; and 2) depletion of donor CD8 T cells eliminates or significantly attenuates sex based differences including both greater female day 14 engraftment and greater male GVH/HVG at day 8. Thus, donor CD8 T cells are not only essential for greater female day 14 engraftment but the greater GVH/HVG in males is also an essential preceding event.

In contrast to the well documented and central role of donor CD4 T cells in inducing a lupus phenotype, the role of donor CD8 T cells is quite the opposite. Donor CD8 T cells typically prevent a lupus phenotype by maturing into effector CTL specific for host MHC I and inducing an acute GVHD phenotype. Conversely, lupus-like disease results when co-injected donor CD8 T

cells fail to completely eliminate host B cells. Such a failure is seen in the DBA→F1 transfer where defects both in the CD8 anti-F1 precursor CTL frequency (15) and the CD4 helper ability have been reported (19). A Similar precursor CTL defect in BALB/c mice is associated with lupus like disease rather than acute GVHD in BALB/c→CB6 F1 mice (20). Lastly, defects in CD8 CTL killing i.e perforin defective donor cells convert acute disease in the B6→BDF1 transfer to lupus-like disease (21). Thus, a critical feature of the present study is that donor CD8 anti-host CTL activity must be sub maximal in order to permit evolution to lupus phenotype. In this setting, our study demonstrates a novel role for donor CD8 T cells in shaping the expression and severity of donor CD4 driven lupus like disease.

The mechanism by which donor CD8 T cells mediate sex based differences in lupus-like disease severity is demonstrated in our studies of donor and host T cell proliferation. Co-injection of donor CD8 T cells induces greater donor CD4 T cell and host CD8 T cell proliferation seen best at day 10 for both sexes. We have previously demonstrated that male DBA→F1 mice exhibit both a parent anti-F1 and an F1 anti-parent cytolytic response. Thus, the earlier GVH and HVG responses shown in this study for males vs females results in a greater degree of donor CD8 T cell contraction in males by day 12-14. The delayed GVH and HVG in females is assocatied with significantly greater donor CD4 T cell proliferation at day 12, greater day 14 CD4 T cell engraftment. Moreover, in individual female CD8 intact→F1 mice, day 14 survival of donor CD8 T cells was correlated with greater donor CD4 T cell engraftment. Taken together, our results support the conclusion that donor CD8 T cells promote further expansion of donor CD4 T cells beyond that induced by host MHC II recognition. The stronger host CD8 and NK downregulatory response at day 8 in males results in greater contraction of donor CD8 T cells in males and a waning of donor CD8 promotion of CD4 T cell proliferation. By contrast, donor CD8 T cells persist at days 12 and 14 in females as a result of the weaker GVH and HVG and are associated with greater numbers of donor CD4 T cells by day 14 in females vs. males.

Footnotes

[1] This work was supported by the following grants (to CSV): National Institutes of Health AI047466 and VA Merit Review. Dr. Puliaev is a recipient of an Engelticheff Fellowship.

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[3] Abbreviations used: GVHD, graft versus host disease; P→F1, parent-into-F1;

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Figure Legends.

Figure 1. Sex based differences in engraftment kinetics of donor CD4 and CD8 T cells in DBA→F1 mice are observed during the second week following transfer. BDF1 mice received unfractionated DBA splenocytes containing either 12 x 10^6 CD4 T cells and 3.9 x 10^6 CD8 T cells (A, C) or 14 x 10^6 CD4 T cells and 4.3 x 10^6 CD8 T cells (B,D) . Recipient F1 mice were sacrificed at the times indicated and donor CD4 (A, B) and CD8 (C,D) T cell engraftment assessed by flow cytometry as described in Methods. Values represent group mean $\pm$ SE (n-= 4-5 mice/group) and results for each time point represent a separate independent experiment. Groups were compared by two-tailed t-test unless otherwise noted. * $p<0.05$; ** $p<0.01$, ***$p<0.001$ for all figures.

Figure 2. Donor CD4 T cell engraftment is directly correlated with donor CD8 T cell engraftment at days 12 and 14 in f→M but not m→M mice. BDF1 mice received unfractionated DBA splenocytes containing 14 x 10^6 CD4 T cells and 4.3 x 10^6 CD8 T cells as described in Figure 1 and linear regression analysis was performed as outlined in Methods. The engrafted numbers of donor CD4 T cells vs. the engrafted number of donor CD8 T cells are shown for individual mice at the indicated time points.

Figure 3. Sex based differences in donor CD4 T cell engraftment are attenuated if donor CD8 T cells are depleted from the inoculum. BDF1 mice received DBA splenocytes depleted of CD8 T cells and containing 14 x 10^6 DBA CD4 T cells. Donor CD4 engraftment was assessed as outlined for Fig. 1 at the indicated time points, each of which represents a separate independent experiment. Values represent group mean $\pm$SE (n= 4-5 mice/group).

Figure 4. Co-injection of donor CD8 T cells is associated with an increase donor CD4 T cells at days 8 and 10. Experimental protocol and groups are as outlined for Figs. 1 and 3 for mice receiving either unfractionated DBA splenocytes containing 14 x 10^6 CD4 T cells and 4.3 x 10^6 CD8 T cells or 14 x 10^6 CD4 T cells depleted of CD8 T cells. The percentage (A, C) and numbers (B,D) of donor CD4 (A, B) and CD8 (C,D) T cells staining KI-67^{hi} were determined by flow cytometry as described in Methods. Values represent group mean $\pm$ SE.

Figure 5. Sex based differences in elimination of host B cells and CD4 T cells are seen at day 8 after transfer. Experimental protocal and groups are as outlined in Figs. 1 and 3. Quantiation of host B cells (A), host CD4 T cells (B) and host CD8 T cells (C) was performed by flow cytometry as described in Methods at the indicated time points.

Figure 6. Co-injection of donor CD8 T cells significantly increases host CD8 T cell proliferation at days 8 and 10. Experimental groups and flow cytometry staining protocol are as outlined for Fig. 4. The percentage (A) and number (B) of host CD8 T cells staining KI-67^{hi} are shown as group mean $\pm$SE for F1 mice receiving either CD8 intact donor cells, CD8 depleted donor cells or uninjected control F1 mice. Representative histograms for KI-67 staining of host CD8 T cells are shown at day 8 (C,D) and day 10 (E,F) for m$\rightarrow$M mice (C,E) and f$\rightarrow$F mice (D,F).

Figure 7. Sex based differences in host NK cells and CD8 T cell upregulation of CD07a are present at day 8. Analysis of host NK cells and CD107 upregulation on donor and host CD8 T

cells was performed at the indicated time points by flow cytometry on the BDF1 cohorts receiving 12 x 10^6 CD4 T cells and 3.9 x 10^6 CD8 T cells described in Fig. 1.

Figure 8. Greater expression of IFN-a inducible genes seen in male CD8 intact→F1 mice is lost with donor CD8 depletion. Cytokine gene expression was measured at the indicated times by RT-PCR as described in Methods for MX-1 (A,B), OAS (C,D) and IP-10 (E, F). Gene expression was determined on total host splenocyte mRNA from either CD8 intact→F1 mice (A,C, E) or CD8 depleted →F1 mice (B,D, F) for the cohorts receiving the 14 x 10^6 dose of donor CD4 T cells shown in Figs 1 B & D and Fig. 3. Values represent group mean ± SE.

Figure 9. Greater expression of IL-21 gene expression in females at day 14 is seen for CD8 intact→F1 mice and lost with donor CD8 depletion. Experimental protocol is as described for Fig. 8. Cytokine gene expression is shown for IL-2 (A,B), IFN-g (C,D) and IL-21 (E,F) for either CD8 intact→F1 mice (A,C, E) or CD8 depleted →F1 mice (B,D, F).

Fig. 1

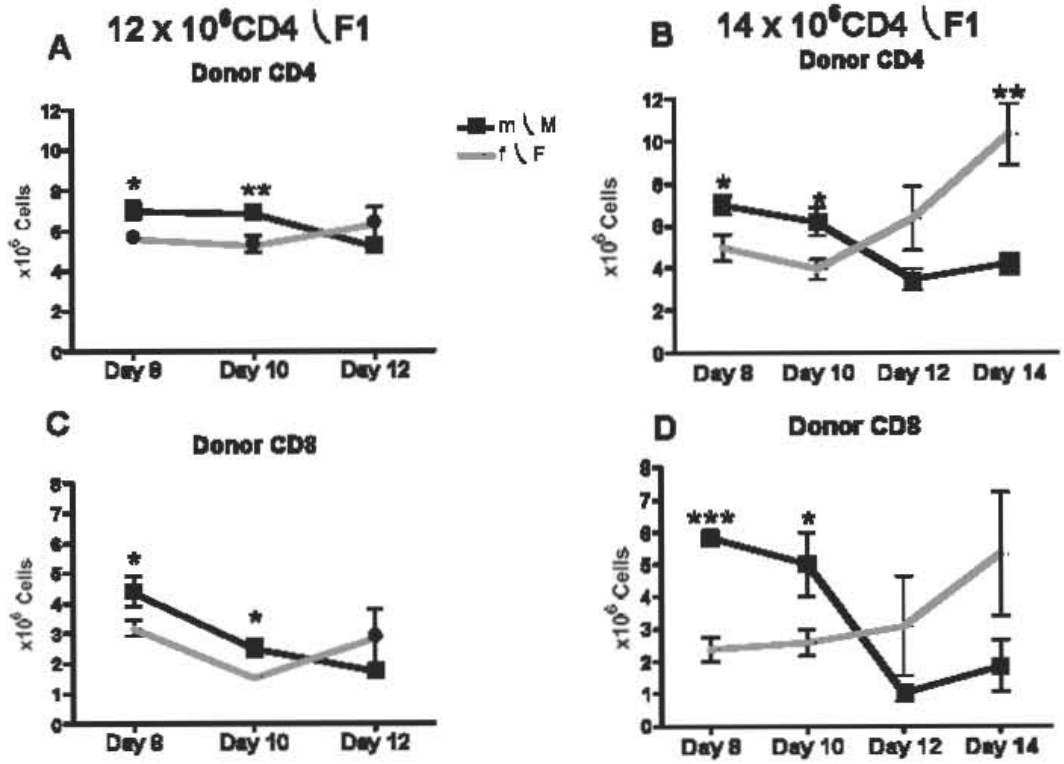

A
12 x 10⁶CD4 \F1
Donor CD4
m \ M
f \ F
x10⁶ Cells
Day 8
Day 10
Day 12
B
14 x 10⁶CD4 \F1
Donor CD4
Day 14
C
Donor CD8
D
Donor CD8

Fig. 2

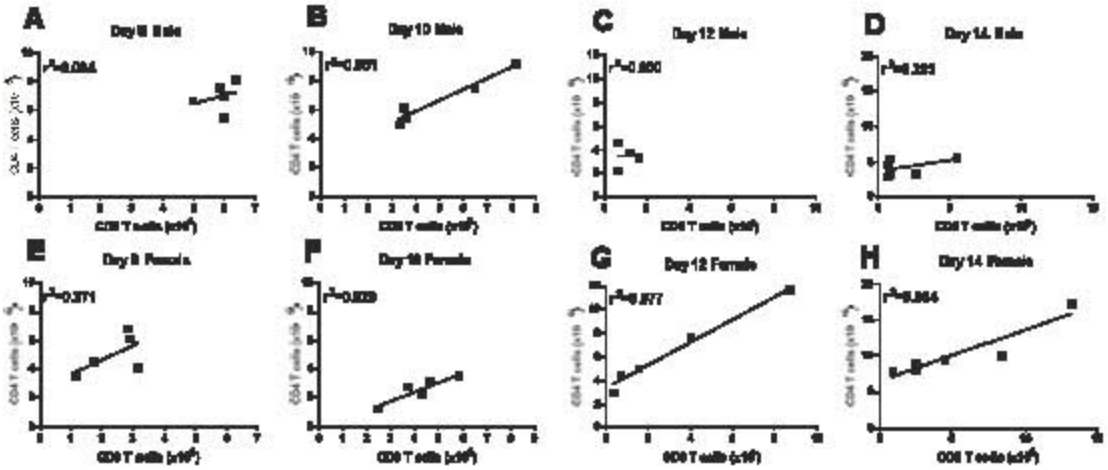


Fig. 3

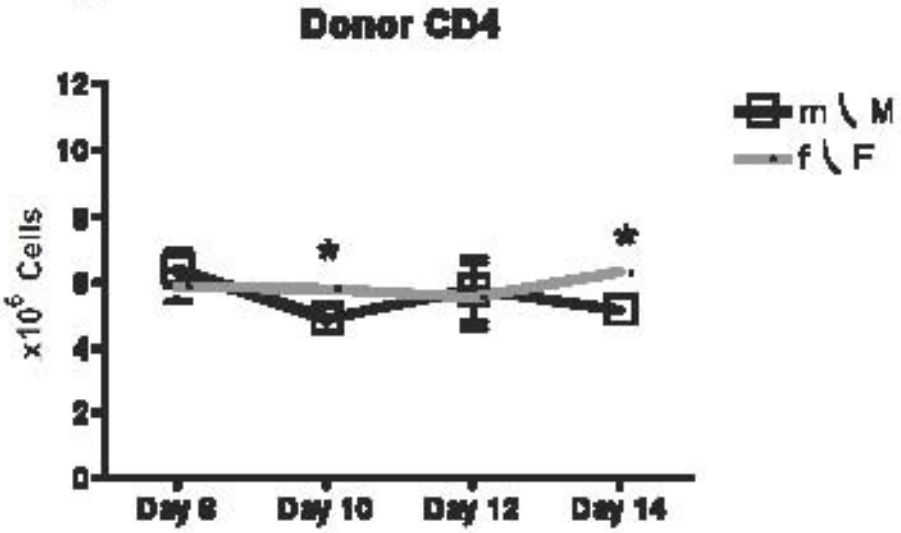


Fig. 4

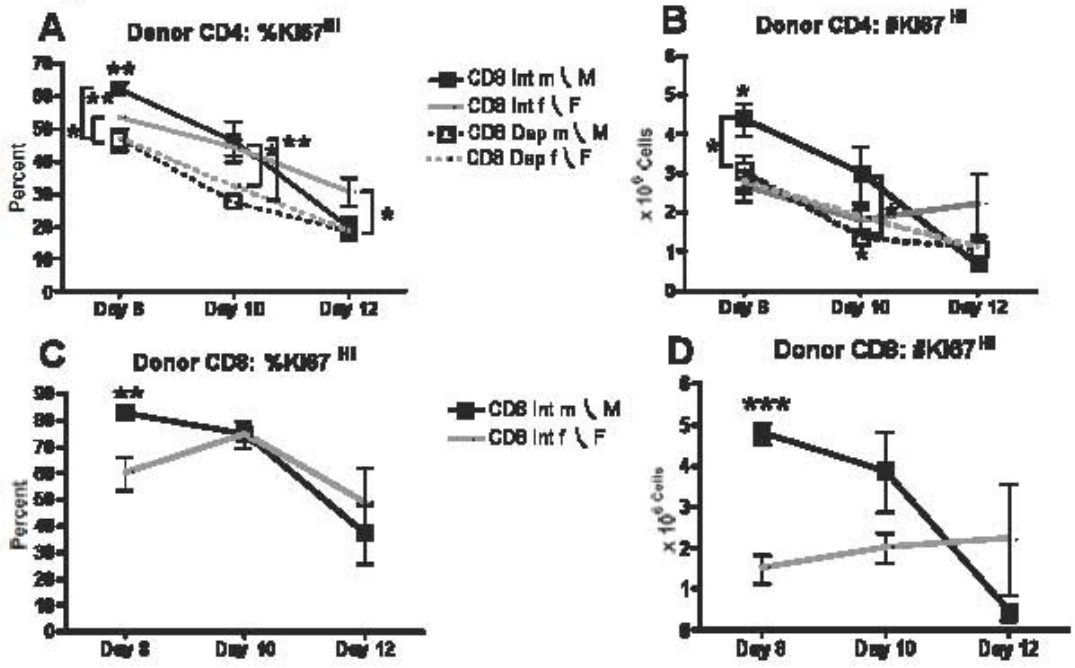

A
Donor CD4: %KI67 HI
Percent
Day 8
Day 10
Day 12
CD8 Int m \ M
CD8 Int f \ F
CD8 Dap m \ M
CD8 Dap f \ F
B
Donor CD4: #KI67 HI
x 10^6 Cells
C
Donor CD8: %KI67 HI
D
Donor CD8: #KI67 HI

Fig. 5

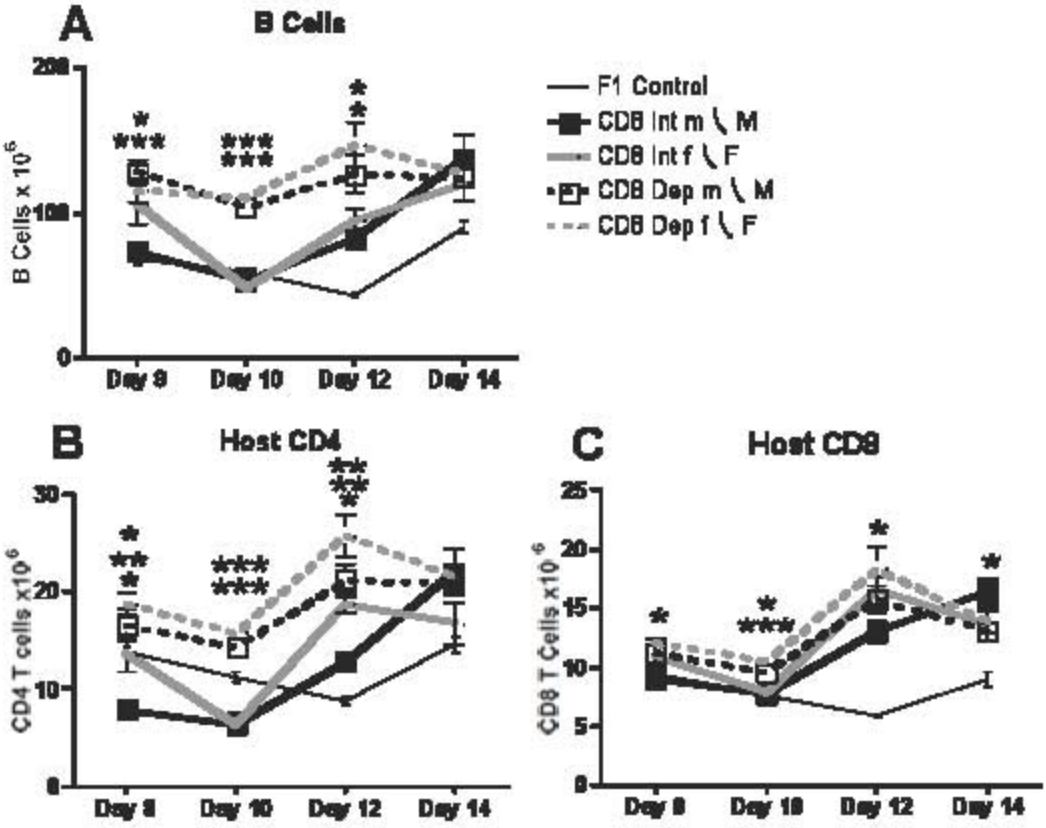

A
B Cells
B Cells x 10^6
Day 9
Day 10
Day 12
Day 14
F1 Control
CD8 Int m \ M
CD8 Int f \ F
CD8 Dep m \ M
CD8 Dep f \ F
B
Host CD4
CD4 T cells x10^6
C
Host CD8
CD8 T Cells x10^6

Fig. 6

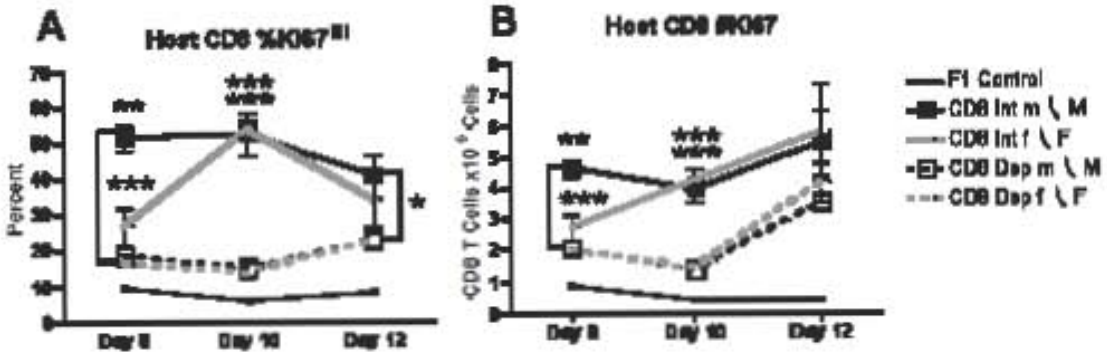

A
Host CD8 %KI67
Percent
Day 8
Day 10
Day 12
B
Host CD8 #KI67
CD8 T Cells x10 6 Cells
F1 Control
CD8 Int m \ M
CD8 Int f \ F
CD8 Dep m \ M
CD8 Dep f \ F

Fig 6

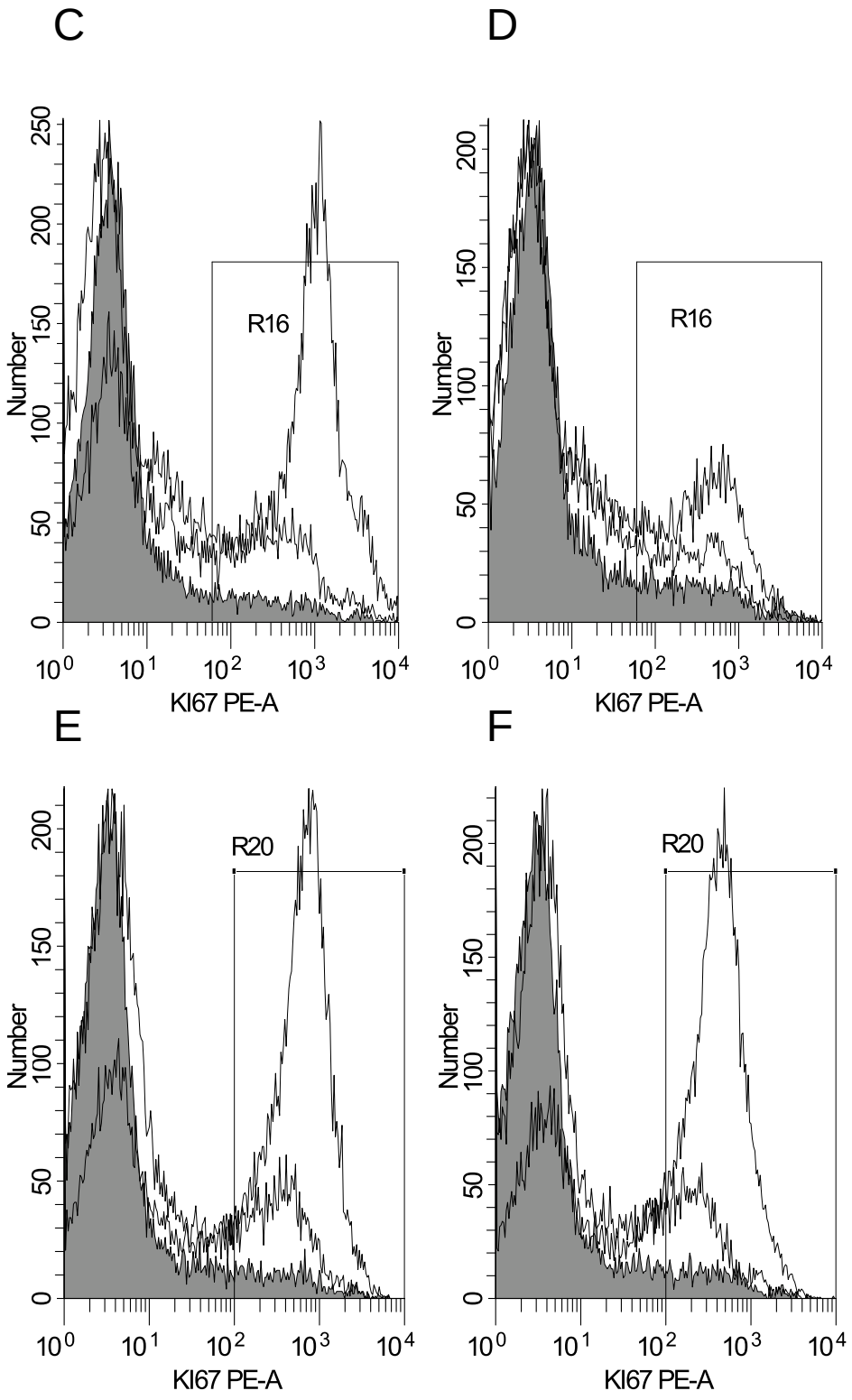

C
D
E
F
R16
R20
Number
KI67 PE-A
0
50
100
150
200
250
10^0
10^1
10^2
10^3
10^4

Fig. 7

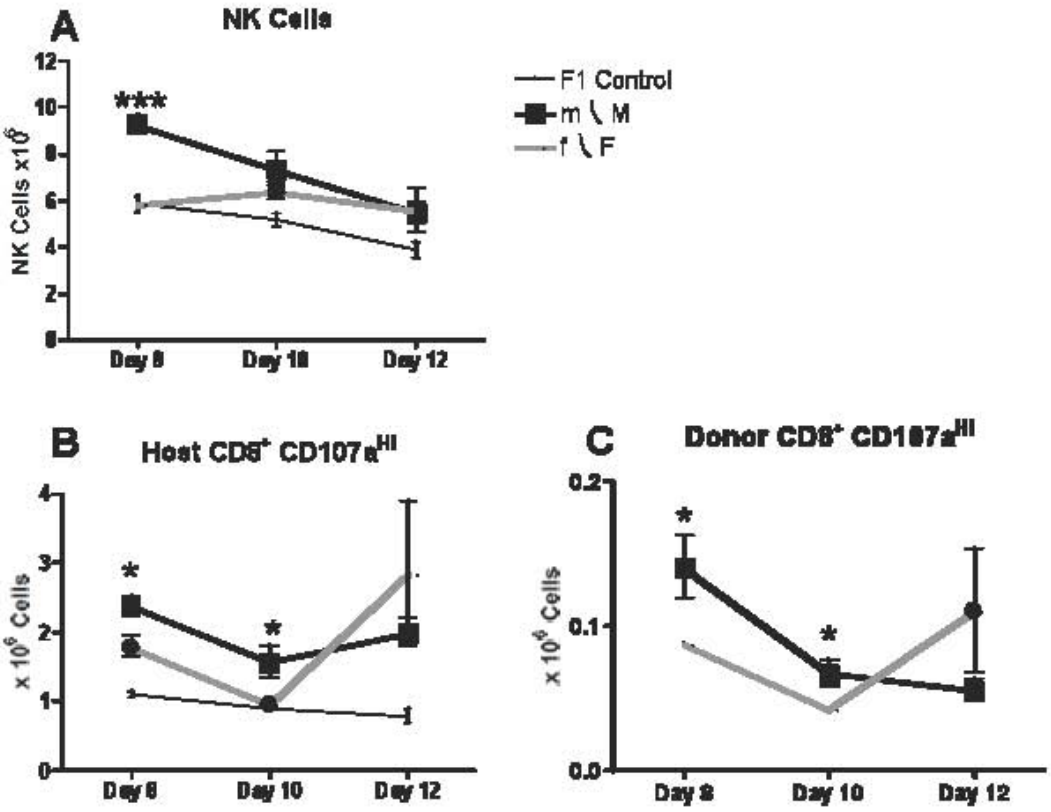

A
NK Cells
NK Cells x10^6
***
Day 8
Day 10
Day 12
F1 Control
m \ M
f \ F
B
Host CD8+ CD107a^HI
x 10^6 Cells
*
*
Day 8
Day 10
Day 12
C
Donor CD8+ CD107a^HI
x 10^6 Cells
*
*
Day 8
Day 10
Day 12

Fig. 8

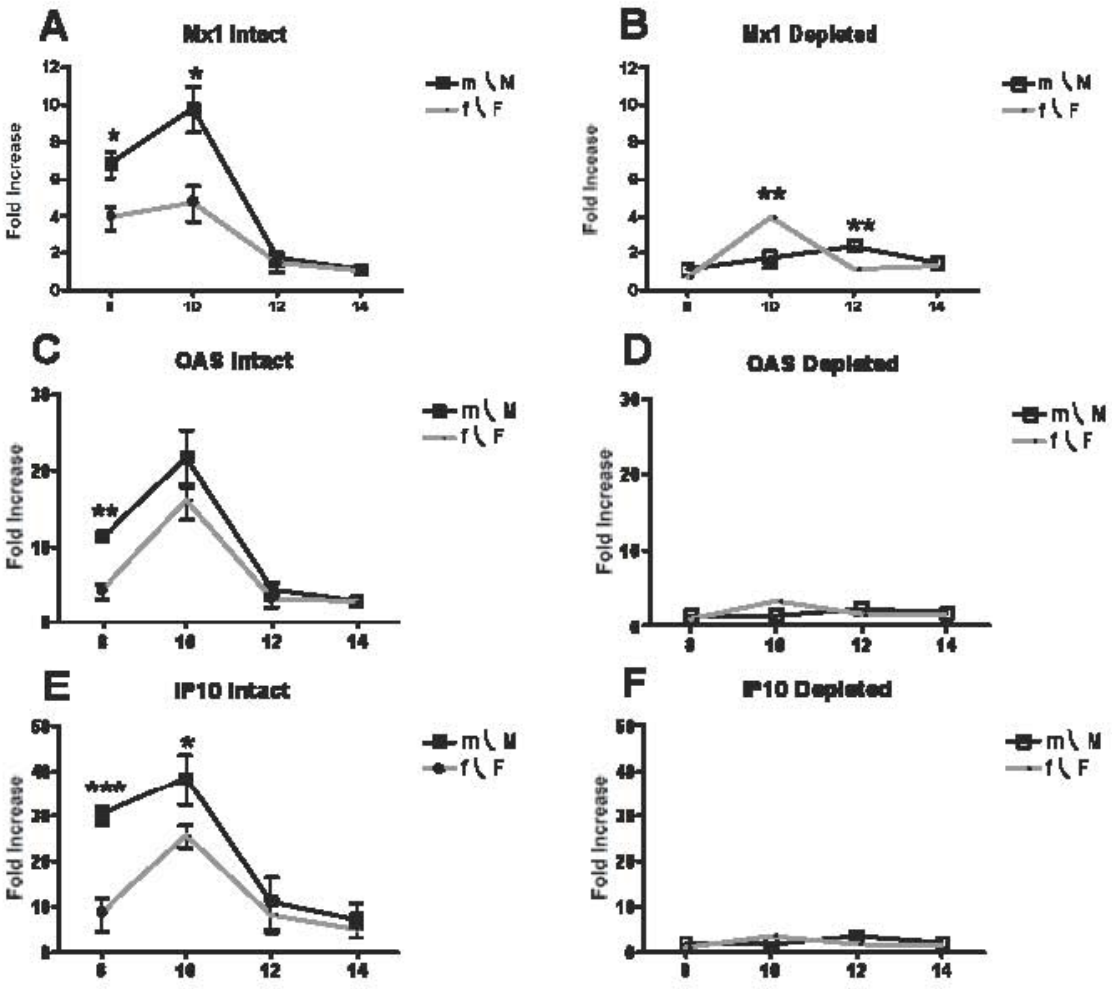
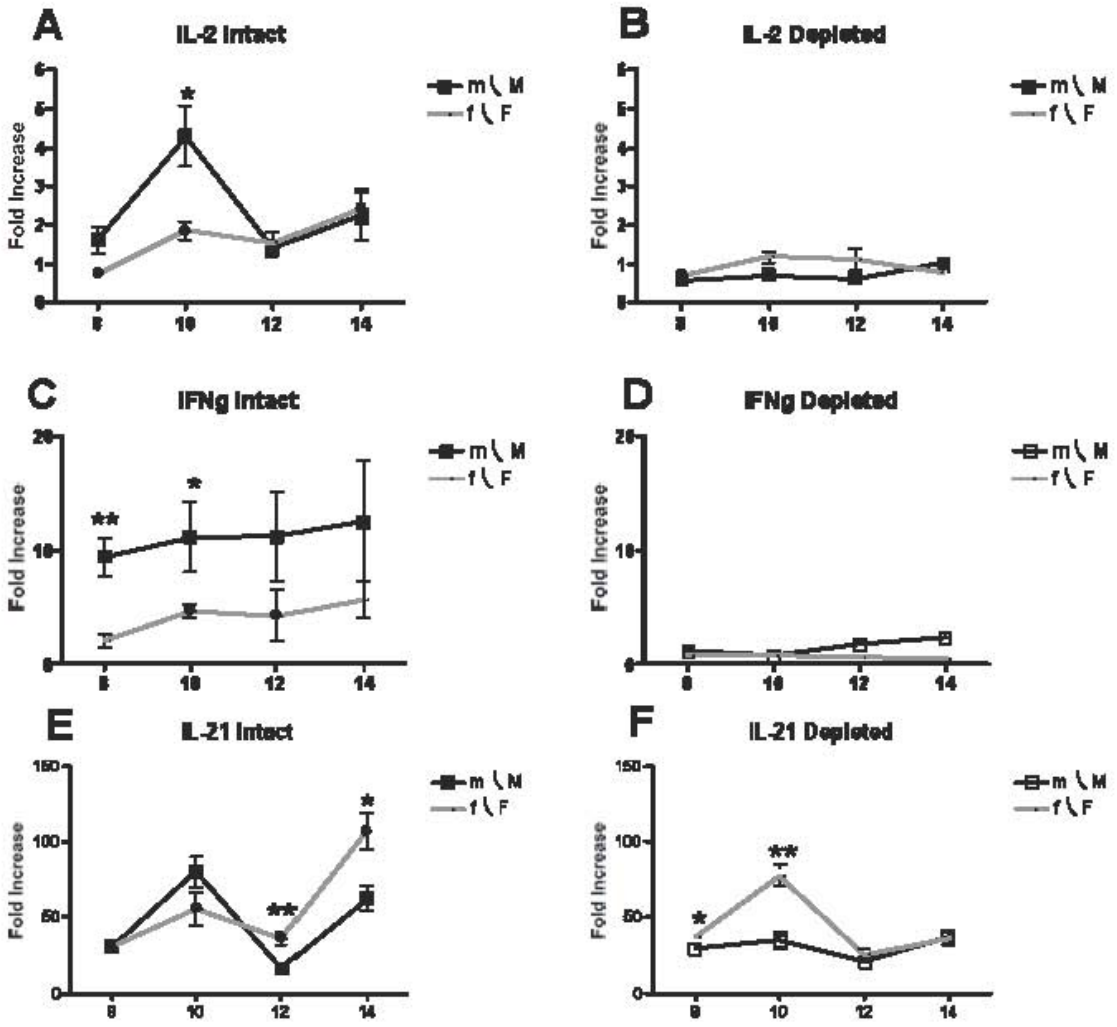

A
Mx1 Intact
Fold Increase
m \ M
f \ F
B
Mx1 Depleted
C
OAS Intact
D
OAS Depleted
E
IP10 Intact
F
IP10 Depleted

Fig. 9


A
IL-2 Intact
B
IL-2 Depleted
C
IFNg Intact
D
IFNg Depleted
E
IL-21 Intact
F
IL-21 Depleted
Fold Increase
m \ M
f \ F
8
10
12
14

Chapter 4: Discussion

This project makes several novel findings regarding murine lupus like disease. Most notably we find that the female predominance in long-term disease severity is dependent upon the presence of $CD8^+$ T cells in the donor inoculum. This is surprising given the necessary and sufficient role of $CD4^+$ T cells in promoting SLE and lupus like disease in mice (1, 2). However, sex based differences in renal disease at 13 weeks were prevented by $CD8^+$ T cell depletion through improvement in f→F mice and a slight worsening in m→M mice. Importantly, lupus like renal disease was not eliminated by injection with $CD8^+$ T cell depleted inocula. As such, donor $CD8^+$ T cells were not critical to the induction of disease but rather for the shaping of sex based differences in the model. The long term study was subsequently followed with short term studies that identify a potential mechanism by which donor $CD8^+$ T cells establish sex based differences in the first 2 weeks of disease progression through 1) the induction of a stronger host versus graft (HVG) response in male hosts and 2) prolonged enhancement of donor $CD4^+$ T cell proliferation and prolonged engraftment of donor $CD8^+$ T cells in female hosts.

The reduction in B cell activity and autoantibody production observed in CD8 depleted f→F vs. CD8 intact f→F mice implicate a reduction in $CD4^+$ T cell help to autoreactive B cells. While $CD8^+$ T cell depletion prior to injection did not alter donor $CD4^+$ T cells at 13 weeks, it did affect host $CD4^+$ T cells. CD8 depleted→F1 females had reduced numbers of host $CD4^+$ $ICOS^{Hi}$ T follicular helper (T_{FH}) cells compared with CD8 intact→F1 females at 13 weeks. This implies that host $CD4^+$ T cells may have a relevant

role in long-term disease pathogenesis and that this population is directly or indirectly affected by the presence or absence of donor $CD8^+$ T cells early in disease induction.

Our initial study using $CD8^+$ T cell depleted donor cells was intended to show that sex based differences in the model were dependent on donor $CD4^+$ T cells and independent of any effect of donor $CD8^+$ T cells. Injection of functional MHC I mismatched $CD8^+$ T cells, in combination with MHC II mismatched $CD4^+$ T cells, results in an acute graft versus host response that destroys the host immune system including B cells. However the DBA $CD8^+$ T cell is well recognized for poor CTL function in this model (3, 4). As such, host B cells are not completely eliminated and, following the normal homeostatic contraction of DBA CTLs, B cells are expanded by donor $CD4^+$ T cells which recognize B6 parental strain Class II expressed on all host B cells. It was therefore expected that if the DBA $CD8^+$ T cell played a role in disease pathogenesis it would be one that inhibited lupus like renal disease, as it does in other p→F1 models (4), through the destruction of autoreactive host B cells. Surprisingly, as we demonstrate, injection of BDF1 mice with $CD8^+$ T cell depleted donor inocula clearly eliminated sex based differences in long term renal disease severity, highlighting a previously unknown but critical role for the donor $CD8^+$ T cell population. Importantly, this included an improvement of disease severity in f→F mice, not a worsening as might be expected. This study finds that CD8 depleted f→F mice exhibit reduced autoantibody deposition in the kidneys, reduced renal disease, and reduced features of nephrotic syndrome as compared with CD8 intact f→F mice. Additionally, sex-based differences for these parameters observed in CD8 intact→F1 mice were lost in CD8 depleted→F1 mice.

A direct role for donor $CD8^+$ T cells in the pathogenesis of lupus like glomerular nephritis is not clear. Previous studies have clearly established that donor $CD4^+$ T cells are necessary and sufficient for inducing lupus like renal disease in this model (5, 6). While one study showed that $CD8^+$ T cells isolated from human lupus patients were capable of stimulating autoantibody production in B cells independently of $CD4^+$ T cells in vitro, these findings have not been corroborated by other studies (7). It is more likely that $CD8^+$ T cells enhance autoantibody production, as indicated by serum anti-ssDNA autoantibody levels, indirectly by enhancing cognate $CD4^+$ T cell help to B cells. At 13 weeks donor $CD4^+$ T cell engraftment was unchanged between CD8 intact→F1 and CD8 depleted→F1 hosts. In both injection cohorts, f→F mice displayed increased engraftment of donor $CD4^+$ T cells compared with m→M mice. However, differences were seen between injection cohorts in host $CD4^+$ T cell populations. This raises two questions: do host $CD4^+$ T cells participate in long-term lupus like disease in the p→F1 model, and secondly how do donor $CD8^+$ T cells can affect the activity of host $CD4^+$ T cells?

It has been previously shown that persistent help from donor $CD4^+$ T cells is necessary for maintenance of long term lupus like disease in the p→F1 model (8). Although donor $CD4^+$ T cells are clearly the population responsible for disease induction, they may not be the only T cells providing help to B cells in long-term chronic GVHD animals. Polyclonal activation of host B cells may in fact result in a phenomenon similar to the epitope spreading seen in SLE patients (9). That is, host B cells activated and expanded by donor $CD4^+$ T cells may then provide stimulation to autoreactive host $CD4^+$ T cells. Similar to host B cells, host $CD4^+$ T cells with moderate affinity for self-targets do survive the process of central tolerance to enter the periphery as mature naïve T cells

(10). Peripheral tolerance normally keeps these cells from expanding and eliciting an autoimmune response. Once that tolerance is broken by donor $CD4^+$ T cell help to autoreactive B cells however, autoreactive host $CD4^+$ T cells may not only proliferate but may provide additional help to B cells in the production of pathogenic autoantibodies.

Participation by host $CD4^+$ T cells in providing help to autoreactive B cells requires their activation and expansion. Consistent with this possibility, splenic host $CD4^+$ T cells in all injection groups demonstrated an increased number of activated T cells and T follicular helper cells (T_{FH}) compared to sex matched uninjected controls. Further, these findings indicate that increased renal disease in CD8 intact f→F mice is associated with increased numbers of host T_{FH} cells compared with CD8 intact m→M mice. This sex-based difference was lost with the use of CD8 depleted injections. There was a slight increase in host T_{FH} in m→M and a slight decrease in f→F that paralleled the slight worsening of disease in CD8 depleted m→M and improvement in CD8 depleted f→F. The long term study therefore suggests that the presence of donor $CD8^+$ T cells in the initial injection, through as yet unknown events, alters the expansion of host $CD4^+$ T cells in a manor that is critical to the establishment of sex-based differences in the model and also infers that host $CD4^+$ T cells provide help to autoreactive B cells in promoting lupus like disease.

At 13 weeks post injection, donor $CD8^+$ T cells were barely detectable in CD8 intact→F1 mice. Any effect they might have on disease outcome would presumably occur in the first 2 weeks prior to homeostatic contraction. The day 14 engraftment of donor $CD4^+$ T cells is an important surrogate marker for long-term disease severity (11). Specifically, using CD8 intact injections, f→F mice exhibit a 2-3 fold greater

engraftment of donor $CD4^+$ T cells compared with that of m→M mice at this time. This was found to predict more severe renal disease in females by 10-12 weeks. Lang et al also demonstrated that the day 14 sex based difference in donor $CD4^+$ T cell engraftment does not occur as a result of differences in apoptotic rate, proliferation from days 0-7, or splenic homing but rather as a result of increased cellular proliferation in females from days 10 to 14. By comparing CD8 intact→F1 and CD8 depleted→F1 injections in short term studies we make the novel observation that, consistent with loss of sex based differences in renal disease, donor $CD8^+$ T cells are required for the 2-3 fold increase in donor $CD4^+$ T cell engraftment in f→F mice. While CD8 depleted f→F mice still engrafted significantly more donor $CD4^+$ T cells than CD8 depleted m→M mice there was only a 1.2 fold difference. This occurred mostly as a result of decreased engraftment in CD8 depleted f→F mice, though there was a slight increase in donor $CD4^+$ T cell engraftment for m→M mice compared to CD8 intact m→M. This finding predicts the slight worsening of renal disease in m→M mice and improvement in f→F mice seen in our long term study, and highlights the sensitivity of day 14 donor $CD4^+$ T cell engraftment as a surrogate marker for long term disease severity.

Donor T cell kinetics demonstrate that CD8 intact m→M mice exhibit a stronger donor T cell response than f→F mice at days 8 and 10. This is surprising given the overall female predominance of the model. Increased donor $CD4^+$ and $CD8^+$ T cell engraftment in males was concomitant with greater upregulation of cytokines associated with CTL activity, including IFN-γ, IP10, and the IFN-α inducible genes Mx1 and OAS. Strikingly, the expression each of these cytokines is reduced to near or below control levels in both sexes and at all time points using CD8 depleted injections. Additionally,

increased donor $CD4^+$ T cell engraftment in males at days 8 and 10 are lost in the CD8 depleted cohort. The stronger initial donor T cell response in CD8 intact m→M mice may therefore be relevant to the eventual female predominance of the model.

The DBA→F1 model exhibits a measurable host versus-graft (HVG) response. A stronger HVG response in males is a potential mechanism through which donor T cell engraftment is more effectively downregulated in m→M mice. Analysis of host $CD8^+$ T cell proliferation by KI67 expression demonstrates in fact that m→M mice do elicit a stronger and earlier host CD8 response than females, which was dependent on the presence of donor $CD8^+$ T cells. Host $CD8^+$ T cells in CD8 depleted→F1 mice do proliferate at a greater rate than that seen in uninjected controls, but the rate is significantly lower than that seen in CD8 intact→ F1 mice. The only exception to this was in CD8 intact f→F mice at day 8, which had low engraftment of donor $CD8^+$ T cells and similarly elicited a weak response from the host $CD8^+$ T cells. Days 8 and 10 demonstrate then that the HVG response is not only dependent upon the presence of a donor CD8 population, but also directly related to the size of that population. In a separate kinetics series that used only CD8 intact injections we show that m→M elicit a stronger HVG based on the expression of CD107a on host $CD8^+$ T cells (a marker of degranulation and CTL activity) and increased numbers of host NK cells vs. f→F mice. Host NK cells were included in the study because they produce IFN-γ and may participate in the host anti-parent response. These findings support the conclusion that the more rapid contraction of donor $CD8^+$ T cells in m→M mice occurs as a result of a stronger and earlier HVG response than that seen in f→F.

A stronger male HVG response does not fully explain the reduced donor $CD4^+$ T cells engraftment in CD8 depleted f→F mice vs. CD8 intact f→F mice. Loss of even a weak HVG response in females should either increase donor $CD4^+$ T cell engraftment in females or leave it unchanged. This led us to ask whether the presence of donor $CD8^+$ T cells could alter the rate of proliferation in donor $CD4^+$ T cells. Typically, $CD4^+$ T cells provide help for $CD8^+$ T cells to proliferate and mature into effector CTL. This, however, does not exclude the possibility that $CD8^+$ T cells, through cytokine production or other means, could enhance the proliferation of an already activated $CD4^+$ T cell population. Analysis of KI67 expression on donor $CD4^+$ T cells demonstrates that the presence of donor $CD8^+$ T cells enhances donor $CD4^+$ T cell proliferation in both sexes. At days 8 and 10 CD8 intact m→M exhibit increased proliferation of donor $CD4^+$ T cells compared with CD8 depleted m→M. Similarly, CD8 intact f→F had increased donor $CD4^+$ T cell proliferation compared to the CD8 depleted group at days 10 and 12. As with the host anti-donor response, donor $CD4^+$ T cell proliferation was enhanced not only by the presence of donor $CD8^+$ T cells but varied with the size of the donor $CD8^+$ T cell population. In data not shown, linear regression of individual animals plotting the percent $KI67^{Hi}$ for donor $CD4^+$ and donor $CD8^+$ T cells demonstrates a direct relationship between the proliferation of each population. That is, increased donor $CD8^+$ T cell proliferation correlates with increased donor $CD4^+$ T cell proliferation. This is well demonstrated by examining day 8 and day 12 donor $CD4^+$ T cell proliferation for CD8 intact→F1 mice. As mentioned, donor $CD8^+$ T cell engraftment is higher in m→M at day 8 and in f→F at day 12 (though not significantly). At day 8, donor $CD4^+$ T cell proliferation is significantly higher in m→M compared with both CD8 intact f→F and

CD8 depleted m→M. Similarly at day 12 f→F exhibit greater donor $CD4^+$ T cell proliferation than that seen in CD8 depleted f→F and CD8 intact m→M (though not significantly due to variability). Finally, these findings are consistent with previous work showing that increased day 14 donor $CD4^+$ T cell engraftment in females is due to increased cellular proliferation from days 10 to 14.

The more rapid contraction of donor $CD8^+$ T cells in males may be due not only to a stronger HVG response but also to reduced expression of IL-21 compared with f→F mice. IL-21, in addition to its role in enhancing antibody production in follicular B cells is also important in maintaining $CD8^+$ T cell responses. While it does not increase $CD8^+$ T cell activation or CTL activity, it has been shown to prevent CD8 exhaustion and allow longer proliferation (12, 13). In CD8 intact f→F mice we see that the expression of IL-21 is greater than that seen in m→M at days 12 and 14. This difference was lost in CD8 depleted→F1 mice, though greater expression of IL-21 was seen in females at day 10 for that cohort. This finding provides an additional mechanism through which donor $CD8^+$ T cell engraftment could be maintained longer in female mice. Similarly, long-term studies show that CD8 intact f→F express higher levels of IL-21 vs. m→M. CD8 depletion reduced IL-21 expression, though it did not prevent sex based differences in its expression. Further studies will be necessary to determine if IL-21 plays a critical role in mediating sex based differences in day 14 donor $CD4^+$ T cell engraftment as well as in long term renal disease.

This project identifies a new potential mechanism through which the female predominance in murine lupus like disease occurs. In both male and female hosts, donor $CD8^+$ T cells enhance the proliferation of the donor $CD4^+$ T cells that drive lupus like

disease. As a result of a stronger HVG response in male hosts and/or increased IL-21 expression in female hosts, donor $CD8^+$ T cell engraftment is maintained longer in f→F mice. Further studies (discussed below) will be necessary to determine precisely how donor $CD8^+$ T cells influence the proliferation of donor $CD4^+$ T cells. In males the donor $CD8^+$ T cell GVH response occurs earlier and is stronger. The greater production of CTL associated cytokines in m→M mice may assist host $CD8^+$ T cells in the HVG response. Conversely, in f→F mice donor $CD8^+$ T cell proliferation is low at day 8 and continues after peak donor CTL activity, which occurs at day 10 in the DBA→F1(14, 15). Because donor $CD8^+$ T cells continue to proliferate after CTL activity has been reduced in females, there are reduced levels of IFN and IFN induced genes, possibly limiting the HVG response.

It is not known what antigens initiate SLE in humans. The p→ F1 model is useful in that it allows for the direct analysis of those T cells that break tolerance and initiate lupus like disease in a normally functioning murine immune system. Our findings predict that in the female environment weaker CTL responses are more slowly downregulated compared to that seen in the male environment. This $CD8^+$ T cell activity enhances the proliferation and function of the $CD4^+$ T cells that break tolerance and stimulate autoreactive B cell activation and proliferation. Conversely in males, a stronger CTL response is more readily shut down and prevents assistance to those $CD4^+$ T cells. IL-21 may play a critical role not only in long term signaling to B cells, but also in maintaining transient $CD8^+$ T cell activation in females. The mechanism through which $CD8^+$ T cells assist the proliferation and activity of $CD4^+$ T cells requires additional study to be fully understood. However, such events may not be isolated to the initial activation of lupus or

murine lupus like disease. Polyclonal activation of B cells in lupus patients leads to the activation of additional self-reactive $CD4^+$ T cells, and we provide associative data that a similar process occurs in the p→F1 model. Once activated and expanded, autoreactive $CD4^+$ T cells would then be capable of providing help to autoreactive $CD8^+$ T cells. In that scenario, a process similar to that which we demonstrate in our short-term studies may occur, wherein host $CD8^+$ T cell activity assists the proliferation of host autoreactive $CD4^+$ T cells and this process is prolonged in females.

Implications for Other Findings on Sex-Based Differences in Lupus

As discussed in the introductory section, sex hormones have been clearly implicated in the female predominance of SLE and in murine models of lupus that exhibit sex-based differences. Likewise, it is probable that the sex-based differences in $CD8^+$ T cell function reported here result from hormonal differences in m→M vs. f→F mice. However, until a precise mechanism for the role of donor $CD8^+$ T cells is established, it would be premature to undertake experiments involving the manipulation of sex hormones in this model.

Another group has forwarded a hypothesis for the cellular mechanism behind the female predominance of murine lupus-like disease. As mentioned previously, Diamond et al have demonstrated that autoreactive B cells are more resistant to apoptosis following exposure to estrogen (16, 17). These findings indicate that increase severity of lupus-like disease in female mice results from prolonged survival of autoantibody producing B cells vs. males. This hypothesis is not mutually exclusive from the hypothesis described here. That is, prolonged B cell survival may be a secondary result of enhanced $CD4^+$ T cell

help that differs between male and female murine lupus. Indeed, our findings demonstrate that long-term sex based differences in disease severity are associated with increased B cell numbers and increase B cell activity in f→F vs. m→M mice. However, Diamond et al indicate that the estrogen treated B cells that resisted apoptosis had a marginal zone (MZ) phenotype. These findings are not in keeping with the general consensus that pathogenic B cells in SLE are T cell dependent and have a germinal center phenotype (18). Similarly, we have observed that long-term disease in the cGVHD model is associated with a reduction in MZ B cells and an increase in follicular B cells compared to uninjected controls in both m→M and f→F mice (Foster, AD unpublished observations).

Limitations to Model:

The use of any animal model of disease has limitations. By definition they are "models" of a disease, and not the disease itself. Despite many similarities, human physiology and murine physiology clearly differ in relevant ways. For example, the p→F1 model of lupus, like other murine models, does not exhibit the typical "waxing and waning" of disease activity seen in human SLE. Rather, disease activity in mice is constant and becomes progressively worse until it eventually plateaus or results in death. The reason for this difference in disease activity may relate to differences in the antigen, that initiates the disease. In the DBA→F1 model, donor T cells respond to alloantigen, which is constantly present. The antigen that initiates SLE is unknown, though some speculate it is EBV or another virus. In this case the initial antigen would only be transiently present. Flares in disease may then result from renewed exposure to the

antigen that initiated disease. The distinctions between a constantly present antigen vs. a transiently present antigen may have additional ramifications on disease expression. Regardless, this murine model is not suitable for the study of disease flares. Additionally, the DBA→F1 model only reflects one aspect of SLE, which is lupus nephritis. It does not model cutaneous lupus, involvement of the central nervous system, or other features of SLE beyond glomerular nephritis.

The breaking of peripheral tolerance is a critical step in lupus pathogenesis that is poorly understood. The use of an induced lupus model prevents the study of the precise mechanism by which tolerance is lost. In the p→F1 model, peripheral tolerance is broken through the artificial addition of Class II mismatched parental strain $CD4^+$ T cells. A spontaneous model of SLE would be more useful for the study of peripheral tolerance in lupus. However, the focus of this project was to analyze sex based differences in disease. Once tolerance is broken, f→F mice develop a more severe disease than m→M indicating that T and B lymphocytes respond differently to the same stimuli in the male environment vs. the female environment. It is unlikely that peripheral tolerance is broken by different mechanisms in the male vs. female immune system.

The DBA→F1, like the NZBWF1 model, displays a female predominance in severity. However in humans, sex-based differences are seen in the prevalence of the disease rather than the severity. We are unable to directly infer why the female immune system is more likely to initiate an autoimmune response than the male immune system since all experimental animals develop a lupus like disease. Rather, murine models of SLE that display sex based differences demonstrate how T cell and B cell responses are shaped differently in the male versus female environment. The DBA→F1 model in

particular, allows for such an analysis in a normally functioning immune system that lacks the genetic defects seen in NZB/W F1 mice. With respects to this study, if we are able to define the precise mechanism by which donor $CD8^+$ T cells mediate sex based differences this may provide insights that can be inferred back to the human disease. It may also identify novel therapeutic targets that are responsible for worsening or improvement of disease.

The DBA/2 strain has a well-characterized defect in CTL responses (3, 4). It is unclear if or how this could limit the DBA→F1 model of cGVHD. As mentioned, it is unknown what events initiate SLE. Presumably, a population of $CD4^+$ T cells breaks peripheral tolerance by providing help to autoreactive B cells. This study presumes that the initial pathogenesis is not isolated to interactions between $CD4^+$ T cells and B cells alone, but includes some aspect of $CD8^+$ T cell mediated cellular immunity. An ineffective CTL response against viral infection may in fact be a critical step in the initiation of lupus. In that case, the defective CTL response of the DBA into F1 model would reflect the human disease. However, it is difficult to speculate how the DBA CD8 defect limits or benefits the interpretation of these findings.

Future Directions

Additional studies will be necessary to better understand the relevance of these findings. First and foremost it will be necessary to find the precise mechanism by which donor $CD8^+$ T cells mediate sex based differences in donor $CD4^+$ T cell engraftment at day 14 and subsequently in long term disease for the DBA→F1 model. Since the cytokine expression varied between CD8 depleted→F1 and CD8 intact→F1 mice at days

8-14, a kinetics study of days 1-7 should provide useful information. Cytokine blockade would also help to determine the role of individual cytokines in this process. Candidates include the Th1 associated cytokines IFN-γ and IFN-α. IFN-γ serves different roles at different time points of the T cell response. In the early stages it is important for both $CD4^+$ and $CD8^+$ T cell activation (19). However, later in the response it is critical to activation induced cell death (20). Given the drop in IFN-γ transcript production at day 8 in CD8 depleted→F1 mice, IFN-γ blockade at day 6 or 7 should determine its role in sex based differences whereas blockade at day 2 or 3 may reduce T cell responses in both sexes. Also, IL-21 was associated with long-term sex based differences in our study and has recently been shown to prolong CTL responses (12, 13). If IL-21 is critical to differential donor $CD8^+$ T cell survival, its blockade prior to day 8 should prevent prolonged survival in f→F mice. This should in turn prevent or reduce the 2-3-fold increase in day 14 donor $CD4^+$ T cell engraftment normally seen in females and, by extension, long term differences in disease severity.

The long-term effect of the DBA $CD8^+$ T cell may also be confirmed by the transfer of purified donor $CD8^+$ T cells into F1 mice with active cGVHD. It has been previously demonstrated that donor $CD4^+$ T cells maintain help to host B cells in the DBA→F1 model up to 10 weeks post-transfer (8). It is not known whether DBA $CD4^+$ T cells maintain their limited ability to provide help to $CD8^+$ T cells. However, the transfer of unstimulated donor $CD8^+$ T cells into CD8 depleted→F1 mice at several different time points would help elucidate its role in sex-based differences for the model. One question is whether they enhance donor $CD4^+$ T cell function during the initial T cell activation phase (days 0-7) or during the mild CTL phase (days 8-10). By day 8, donor $CD4^+$ T

cells have already become activated effector T cells. If the role of donor $CD8^+$ T cells is in promoting $CD4^+$ T cell proliferation during the CTL phase and/or assisting in T cell downregulation at that time, then transfer of donor CD8 T cells into CD8 depleted→F1 mice at day 7 should "rescue" sex-based differences. If sex-based differences are not restored this would suggest that the presence of donor $CD8^+$ T cells is important during initial $CD4^+$ T cell activation (days 0-7). One possible complication for such a study would be the role of donor $CD4^+Foxp3^+$ Tregs. Previous studies in our lab have shown that donor Treg downregulation occurs simultaneously with donor $CD8^+$ T cell downregulation, and that CD8 depleted→F1 mice have a trend towards reduced percentage, number, and proliferation of donor Tregs at days 8-12 compared with CD8 intact→F1 mice (Foster, AD unpublished observations). Also, depletion of donor $CD25^+$ cells prior to injection enhances donor anti-host CTL responses, preventing lupus-like cGHVD and generating an acute-like response (21). As such, injection of unstimulated $CD8^+$ DBA T cells into CD8 depleted->F1 mice at day 7 may actually reduce or prevent lupus-like disease by killing host B cells if donor $CD4^+$ T cell help to $CD8^+$ T cells is enhanced due to reduced Treg activity.

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